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REMARKS

I. Status of the Claims

With entry of this Amendment, claims 47, 61-75, 78, 79, and 83 are pending in the application. All claims have been rejected.

Applicants have amended claim 47 solely to more clearly recite their invention. Support for the proposed amendment is found in the specification and claims as originally filed. For example, expression of superoxide dismutase according to the invention for treatment of Parkinson's disease is supported in original claim 17. Applicants have also amended claim 83. The amendments do not add any new matter.

Applicants have canceled without prejudice or disclaimer claims 1-46, 48-60, 76, 77, and 80-82. They reserve the right to pursue the subject matter recited in those claims in a divisional or continuation application.

II. The Specification Enables the Full Scope of the Claims

All claims are rejected under 35 U.S.C. § 112, first paragraph, because they allegedly contain subject matter that was not described in the specification in such a way as to enable one skilled in the art to make or use the invention without undue experimentation. Office Action, pages 2-7. Specifically, the Office alleges:

> [T]he specification merely describes putting the SOD-1 gene into an Ad vector, and prophetically claims its ability to serve as an adequate gene therapy device. There are no working examples of a therapeutic use for the vector, and there is no indication that such a vector has nay effect in a model system for any of the diseases it is claimed to treat.

Office Action, page 6. Furthermore, The Office alleges that "[t]he State of the Art, even at post-filing to the instant application, indicates that there are no Ad vectors that can adequately sustain the expression of a therapeutic gene in a human." *Id.* Applicants

respectfully traverse the rejection.

Post-filing Evidence of Enablement

Solely to advance prosecution, Applicants have amended the claims so that they now all recite "[a] method comprising administering to a Parkinson's disease patient a replication defective, recombinant adenovirus comprising a DNA sequence which encodes an intracellular CuZn superoxide dismutase-1 (SOD-1), wherein the DNA sequence is under the control of a signal enabling expression in a target cell and wherein said expression of said superoxide dismutase results in a treatment of Parkinson's disease." Applicants submit that the specification enables the claims as amended.

The efficacy of the recombinant defective adenovirus encoding SOD (superoxide dismutase) on Parkinson's disease as presently claimed and described has been subsequently confirmed and validated in several post-filing scientific articles of the inventors. Applicants enclose copies of three articles:

(1) Barkats *et al.*, *Neuronal Transfer of the Human Cu/Zn Superoxide Dismutase Gene Increases the Resistance of Dopaminergic Neurons to 6-hydroxydopamine*, J. Neurochem., 82:101-109 (2002) ("Barkats-2002");

(2) Barkats *et al.*, *Adenovirus in the Brain: Recent Advances of Gene Therapy for Neurodegenerative Diseases*, Progress Neurobiol., 59:333-341 (1998) ("Barkats-1998"); and

(3) Barkats *et al.*, *An Adenovirus Encoding CuZnSOD Protects Cultured Striatal Neurons Against Glutamate Toxicity*, Neuropharm. Neurotox., 7:497-501 (1996) ("Barkats-1996").

In Barkats-2002, the inventors showed that the survival of the dopaminergic cells

exposed to 6-OHDA was 50% higher among the SOD1-producing cells than cells infected with control adenoviruses. That evidence demonstrates that the survival of the dopaminergic neurons can be highly increased by the adenoviral gene transfer of SOD1. This effect supports the beneficial use of this vector for protecting neurons in Parkinson's disease. *See* Abstract.

In Barkats-1998, the inventors confirmed that, in a rat model of Parkinson's disese, adenoviruses encoding superoxide dismutase improved the survival and functional efficacy of dopaminergic cells. *See* Abstract and Section 3.1.1 at page 336. They also confirm in this review that sustained expression can be obtained with the adenoviral vector according to the present invention. "Human SOD-1 and B-galactosidase were produced in the grafts four days after transplantation and the expression persisted at 5 weeks thereafter." Section 3.1.1 at page 336. Furthermore, a minimal immunological response was observed with use of the Ad-SOD vector. "The inflammatory consequences of the adenovirus gene transfer were minimal and only a moderate microglial response around the graft tissue was detected...." *Id.*

In Barkats-1996, it was shown that infection of striatal cells with a recombinant adenovirus expressing SOD protected those cells from glutamate-induced cell death (*see* Abstract) supporting the therapeutic application of this vector for a wide range of neurodegenerative disorders, including Parkinson's disease.

The Office concludes that "the instantly claimed invention is not enabled because there is no currently known way to sustain the expression of a therapeutic gene in a human, by using an adenoviral vector." Office Action, page 7. As a basis for this conclusion, the Office asserts that:

> In order to practice the instantly claimed invention, the skilled artisan would be forced to practice an incredible amount of undue and unpredictable trial and error experimentation. The State of the Art, even at post-filing to the instant application, indicates that there are no Ad vectors that can adequately sustain the expression of a therapeutic gene in a human. This is because of the elicitation of a strong immune response in the cells upon delivery of the vector, which subsequently results in decreased expression of the therapeutic gene.

Id., page 6. The evidence presented herein, however, clearly rebuts the Office's position. As discussed above, Barkats-1996, Barkats-1998, and Barkats-2002 show that the methods of administering Ad to deliver an SOD gene disclosed in the Applicants' specification result in sustained expression of SOD-1, minimal immune response, and increased cell survival. The experimental methods in these references relied upon the methods disclosed in the Applicants' specification to reach their subsequent results. This evidence supports Applicants' arguments that the present claims did not require undue and unpredictable trial and error experimentation to be practiced at the time of filing, as asserted by the Office. Thus, contrary to the Office's assertion, the specification was enabling to those of skill in the art as of the filing date, as evidenced by the subsequent publications, particularly in regard to administration of Ad to deliver an SOD gene, and its subsequent expression for use in treating neurodegenerative diseases and disorders, including Parkinson's disease. See MPEP § 2164.05(b).

References Cited by the Office

The Office cites Verma *et al.*, Nature 389:239-242 (1997) ("Verma"); Anderson, Nature 392:25-30 (1998) ("Anderson"); Mountain (TIBTECH 18:119-128 (2000) ("Mountain"); and Check, Nature 421:305 (2003) ("Check") and asserts the following reasons why it believes undue experimentation is required to practice the invention.

Verma and Anderson are cited by the Office as evidence that adenoviral vectors have difficulty in producing sustained expression for more than 5-10 days, and that immunological response to the vector is partially to blame for this deficiency. Office Action, page 4. As discussed above, Barkats-2002, Barkats-1998, and Barkats-1996 provide specific evidence that the specification enables the claimed method for administering Ad-SOD to a patient, expressing SOD, and treating Parkinson's disease in the patient. In particular, as discussed above, sustained expression of SOD was achieved for 5 weeks and a minimal immunological response was observed with use of the Ad-SOD vector. Barkats-1998, Section 3.1.1 at page 336. Neither Verma nor Anderson cite any Barkats references.

Check is cited by the Office as evidence of a high degree of unpredictability of treatment. *Id.*, page 5. Applicants, however, note that Check refers only to retroviral vectors, and makes no mention of the unpredictability of specifically using adenoviral vectors. Check is therefore not germane to the enablement of the current claims which recite the use of adenovirus.

Further, Applicants submit that the disclosures of Mountain, Verma, Anderson, and Check regarding the problems and obstacles to achieving efficacious or successful gene therapy are merely referring to safety considerations inherent in the clinical trial regulatory process generally, and are not addressing the enablement requirement of whether the skilled artisan could practice the specific methods recited in the claims without undue experimentation upon reading the specification. For example, Mountain and Anderson review various clinical studies and stress the importance of the approval process required by the Recombinant Advisory Committee as well as the Food and Drug Administration who assess any possible safety risks of new therapies by requiring

clinical trials. Mountain Page 126; and Anderson, page 29. Evidence of the general safety of gene therapies is not germane to the issue of enablement of the present claims.

In addition, the Office has provided no basis for concluding that the relied-upon generalized problems with gene therapy would prevent the skilled artisan from practicing the specific claimed method. The experimental data in the specification, and subsequently supported by Barkats-2002, Barkats-1998, and Barkats-1996, clearly indicate successful Ad delivery and expression of an SOD gene useful for the treatment of Parkinson's disease. Expression was sustained for at least 5 weeks and the alleged disadvantageous high immunogenicity of adenoviruses did not present a problem in the inventors' experiments.

In view of these remarks, Applicants submit that proposed amended claims meet the requirements of section 112, first paragraph. Reconsideration and withdrawal of the rejection is respectfully requested.

III. CONCLUSION

In view of the foregoing, Applicants respectfully request that the Office reconsider and withdraw the enablement rejections of pending claims 47, 61-75, 78, 79, and 83, and allow all pending claims.

Please grant any extensions of time required to enter this amendment and response and charge any additional required fees to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
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Dated: Monday, July 26, 2004

By: _____
Charles D. Niebylski
Reg. No. 46,116

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An adenovirus encoding CuZnSOD protects cultured striatal neurones against glutamate toxicity

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SUPEROXIDE dismutase (SOD), a key enzyme in the detoxification of free radicals, catalyses the dismutation of superoxide $O_2^{\bullet-}$ to oxygen and hydrogen peroxide (H_2O_2). It is therefore a promising candidate for gene transfer therapy of neurological diseases in which free radicals are thought to be involved. We have constructed a recombinant adenoviral vector containing the human copper–zinc SOD cDNA. Using this vector we were able to drive the production of an active human copper–zinc SOD protein (hCuZnSOD) in various cell lines and primary cultures. Infection of striatal cells with a recombinant adenovirus expressing hCuZnSOD protected these cells from glutamate-induced cell death.

Key Words: Superoxide dismutase; Recombinant adenovirus; Striatal neurones; Glutamate; Excitotoxicity; Oxidative stress

Introduction

Accumulating evidence indicates that excessive formation of free radicals may be involved in the pathophysiology of many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis.[1-3] Oxidative stress is also implicated in acute brain disorders such as ischaemia and traumatic damage.[4] Both enzymatic and non-enzymatic systems can down-regulate the levels of oxidants in the brain.[5] Free radicals are quenched by cellular chemical scavengers (such as ascorbic acid in the cytosol or α-tocopherol in membranes), and by an enzyme pathway system consisting of superoxide dismutase (SOD) and catalase/peroxidase. SOD catalyses the dismutation of superoxide ($O_2^{\bullet-}$) to form H_2O_2, which is in turn reduced to water and molecular oxygen by catalase and glutathione peroxidase. SOD is therefore the first step in the enzyme cascade responsible for the detoxification of oxygen-derived free radicals, and increased intracellular levels of SOD may be able to protect neurones against damage by free radicals. A protective effect of endogenous SOD against oxidative damage has been reported in various studies where SOD was overexpressed in transfected cells or transgenic animals. *In vitro*, primary cultures of neurones isolated from copper–zinc SOD (CuZnSOD) transgenic mice were found to be more resistant to glutamate toxicity than controls,[6] and sympathetic neurones microinjected with CuZnSOD were partially protected against the effects of growth factor deprivation.[7] *In vivo*, transgenic mice overexpressing CuZnSOD displayed a lower vulnerability than non-transgenic controls to focal cerebral ischaemic injury,[8] MPTP- and metamphetamine-induced neurotoxicities[9,10] and cerebral infarct after cold injury.[11]

These observations suggest that gene transfer of SOD, leading to overproduction of the enzyme in neurones, may have applications for protection against several forms of neurotoxic insults. An efficient method of gene transfer into brain cells has been developed using recombinant adenoviruses, which are able to infect post-mitotic cells, and in particular neurones.[12] The adenoviral vector has also been shown to be a powerful tool for overproducing proteins of therapeutic interest in neurones *in vitro* and *in vivo*, opening the way to the development of new therapeutic strategies for neurological diseases. We have constructed an adenoviral vector carrying the human CuZnSOD gene (Ad-hCuZnSOD), to be used as a neuroprotecting tool. We report an analysis of whether infection of striatal cells with Ad-hCuZnSOD could reduce the vulnerability of these cells to glutamate-induced injury, as excitotoxicity is involved in a wide range of neurological diseases, and is linked to oxidative stress.[1]

Materials and Methods

Construction of recombinant adenoviruses: The production of recombinant adenovirus expressing the β-galactosidase gene was described previously,[13] and a similar strategy was used to obtain a virus expressing the human CuZnSOD gene (Ad-hCuZnSOD). Briefly, a 622 base pair hCuZnSOD cDNA (a gift from Dr Guy Rouleau, McGill University, Canada) was inserted between the *Pst*I and *Hind* III sites of a Bluescript plasmid (Stratagene) containing the polyadenylation sequence of SV40 in the *Xho*I site. The gene was then prepared as a blunt-ended *Kpn*I/*Sac*I fragment and inserted downstream from the long terminal repeat of the Rous sarcoma virus (LTR RSV) promoter in a shuttle vector containing the inverted terminal repeat (ITR) of the adenoviral genome, encapsidation sequences and adenoviral sequences allowing homologous recombination with the correct part of the viral genome (phCuZnSOD). After linearization by *Xmn*I digestion, phCuZnSOD and the large *Cla*I fragment of Ad-5 DNA were used to co-transfect the transformed human kidney cell line 293 using the calcium phosphate–DNA precipitation method. The transfected cells were overlaid with agar and plaques were screened for the presence of the recombinant adenovirus using enzyme restriction analysis and PCR. Viral stock was prepared by expansion of the recombinant adenovirus in the cell line 293 and purification by ultracentrifugation in a CsCl gradient, followed by dialysis. Virus titers were determined by plaque assays on 293 cells and expressed as plaque forming units (pfu) ml^{-1}. Ad-hCuZnSOD was obtained at a titer of 3×10^{10} pfu ml^{-1}.

Assay of hCuZnSOD enzymatic activity: Mouse NS20Y neuroblastoma cells were infected with Ad-hCuZnSOD at a multiplicity of infection (MOI) of 50 and 100 pfu $cell^{-1}$. Controls were not infected. Human CuZnSOD and host mouse CuZnSOD were identified by gel electrophoresis followed by nitroblue tetrazolium (NBT) staining. In each case a NP-40 extract was prepared from 500 000 NS20Y cells 48 h after infection, loaded on a 15% non-denaturing polyacrylamide gel, and electrophoresis was performed for 3 h at 100 V. SOD was localized by soaking the gel for 20 min in 0.3 mM NBT and 0.26 mM riboflavin, followed by immersion for 20 min in 90 mM tetramethylethylenediamine (TEMED).[14]

Cell culture and infection protocol: Striatal cultures were obtained from E15 Sprague–Dawley rat embryos (Iffa-Credo, France). The lateral ganglionic eminence was dissected out and mechanically dissociated. Cells were seeded on 24-well multiwells at a density of 250 000 cells per well in serum-free medium: DMEM containing penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}; Gibco) and supplemented with 2 mM glutamine, 100 μg ml^{-1} transferrin, 25 μg ml^{-1} insulin, 10 μg ml-1 putrescine, 5 ng ml^{-1} sodium selenite and 6.3 ng ml^{-1} progesterone (all from Sigma). More than 95% of the cells in culture were of neuronal phenotype, as assessed by cellular morphology and immunocytochemical labelling of microtubule-associated protein, β3 tubulin, 160 kDa neurofilament protein and GFAP (data not shown). Infections were performed after 3 or 4 days *in vitro* by changing culture medium for fresh medium containing the virus (Ad-βGal or Ad-hCuZnSOD) at a MOI of 100 or 300 pfu $cell^{-1}$.

Glutamate toxicity: After 6 days *in vitro*, cultures were tested for sensitivity to glutamate. A stock solution of glutamate (Na-salt, Sigma) in DMEM was added directly to the wells to a final concentration of 2.5 mM. The stock solution was prepared in DMEM. Cell viability was determined 24 h later.

Intravital staining of the culture (FDA/PI method): Cells were washed with Locke's solution (154 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 2.3 mM $CaCl_2$, 5.6 mM D-glucose and 8.6 mM HEPES; pH 7.4) and incubated for 5 min at 37°C with a fluorescein diacetate (FDA, 15 μg ml^{-1}) and propidium iodide (PI, 15 μg ml^{-1}) mixture. The medium was then replaced with fresh Locke's solution and cultures were immediately examined under a fluorescence microscope at 488 nm (FDA) and 514 nm (PI).[15] FDA is deesterified only in living cells to produce a green–yellow fluorescence. Neuronal injury facilitates the entry of PI into the cells, and its interaction with DNA produces a red fluorescence. Viable and injured cells were counted from three representative fields for each well (two photographs per field). The percentage of viable cells was computed by assessing the FDA/(PI+FDA) ratio for the three fields.

X-Gal cytochemistry and hCuZnSOD immunocytochemistry: To visualize transgene expression, cells were first fixed for 30 min at 4°C in phosphate buffered saline (PBS) containing 4% paraformaldehyde. β-Galactosidase was detected by incubating the cells for 2 h at 37°C in an X-Gal solution consisting of potassium ferricyanide (4 mM), potassium ferrocyanide (4 mM), MgCl2 (4 mM) and X-Gal (0.4 mg ml^{-1}, Euromedex) in PBS. For SOD immunostaining, mouse NS20Y neuroblastoma cells were incubated for 1 h with PBS containing horse non-specific serum (NSS) and 0.2% Triton X-100, and then for 48 h at 4°C with a monoclonal anti-hCuZnSOD antibody (Sigma) diluted 1/500 in PBS with goat NSS and 0.2% Triton X-100, followed by staining using the vectastain Elite ABC system (Vector laboratories). For striatal neurones, cells were incubated for 1 h in

PBS with 10% pig NSS and 0.2% Triton X-100, and then for 48 h at 4°C with a polyclonal anti-hCuZnSOD antibody (Valbiotech) diluted 1/500 in PBS containing 10% pig NSS and 0.2% Triton X-100, followed by 1 h incubation with biotinylated anti-sheep/goat Ig (1/400, Amersham) and 1 h incubation with streptavidin-biotinylated horseradish peroxidase complex (1/250, Amersham). Both mouse and rat cells were treated with VIP peroxidase substrate (Biosys, France) and hydrogen peroxide. The viability of infected cells was estimated by counting cells expressing the transgene as revealed by X-Gal staining or anti-hCuZnSOD immunochemistry in glutamate-treated and untreated cultures.

Results

A recombinant adenoviral vector expressing the hCuZnSOD gene under the control of a LTR-RSV promoter was obtained. We checked its ability to direct production of a functional enzyme. The enzymatic activity of human CuZnSOD in infected mouse NS20Y neuroblastoma cells was visualized after non-denaturing gel electrophoresis, by nitroblue tetrazolium staining. This assay is based on the generation of superoxide ions reduced by riboflavin, which converts colourless nitroblue tetrazolium into blue formazan.[14] Scavenging of $O_2^{\bullet -}$ by CuZnSOD inhibits colour development, giving rise to a colourless band at the position of the enzyme in the gel. Thus, hCuZnSOD was detected in infected cells (Fig. 1). Endogenous mouse SOD and hCuZnSOD can be discriminated by their different mobilities in the gel (Fig. 1). The band intermediate between mouse and human CuZnSOD presumably corresponds to a heterodimeric form of SOD composed of a human and a mouse subunit. The intensity of the hCuZnSOD band increased with the multiplicity of infection from 50 to 100 pfu cell^{-1}.

Human CuZnSOD was also detected by immunochemistry 48 h after infection by Ad-hCuZnSOD in NS20Y neuroblastoma cells (Fig. 2), in PC12 cells and in primary culture cells of striatum (Fig. 3C,D), cortex, cerebellum and mesencephalon (results not shown), using an antibody able to discriminate between the endogenous rodent SOD and the exogenous human form of the enzyme. The immunoreactive cells were uniformly stained, showing that the recombinant protein was present throughout the cytoplasm. The efficiency of infection with Ad-hCuZnSOD varied among the different cell types. A MOI of 300 pfu cell^{-1} for striatal neurones in primary culture led to approximately 10% of cells expressing the transgene. We investigated the neuroprotective capacities of Ad-hCuZnSOD against glutamate toxicity in striatal cells. Cells were infected with Ad-βGal or Ad-hCuZnSOD at a MOI of 100

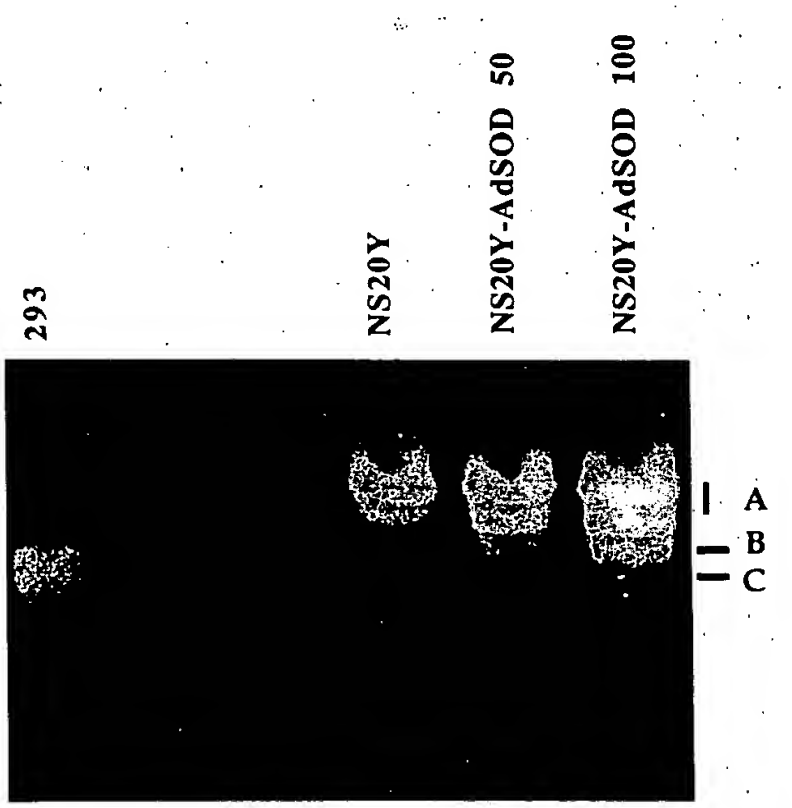


FIG. 1. Enzymatic activity of hCuZn-SOD in mouse NS20Y neuroblastoma cells non infected (lane 2) or infected with 50 and 100 pfu. cells^{-1}(lanes 3 and 4 respectively). The position of human CuZnSOD activity band is shown for uninfected human 293 cells (lane 1). (A) mouse CuZnSOD; (B) hybrid heterodimer; (C) human CuZnSOD.

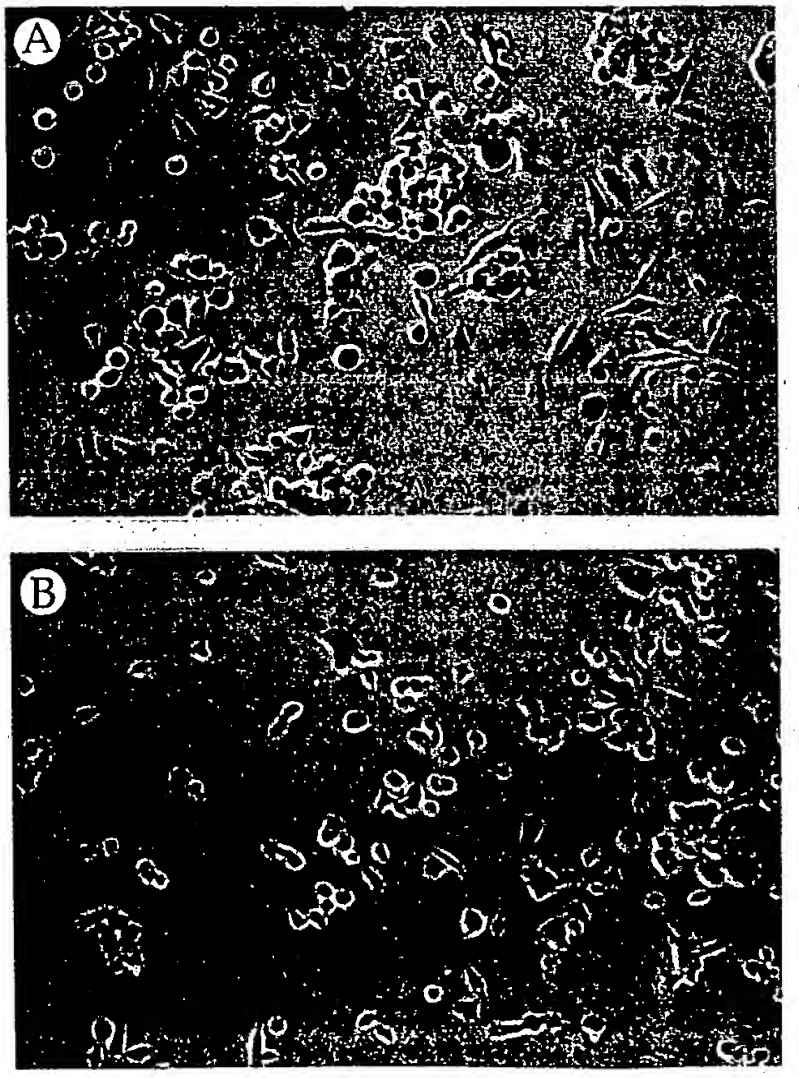


FIG. 2. Immunodetection of hCuZnSOD in cell line NS20Y. Cells were fixed for immunostaining 48 h after infection with Ad-hCuZnSOD. (A) infected cells; (B) uninfected cells.

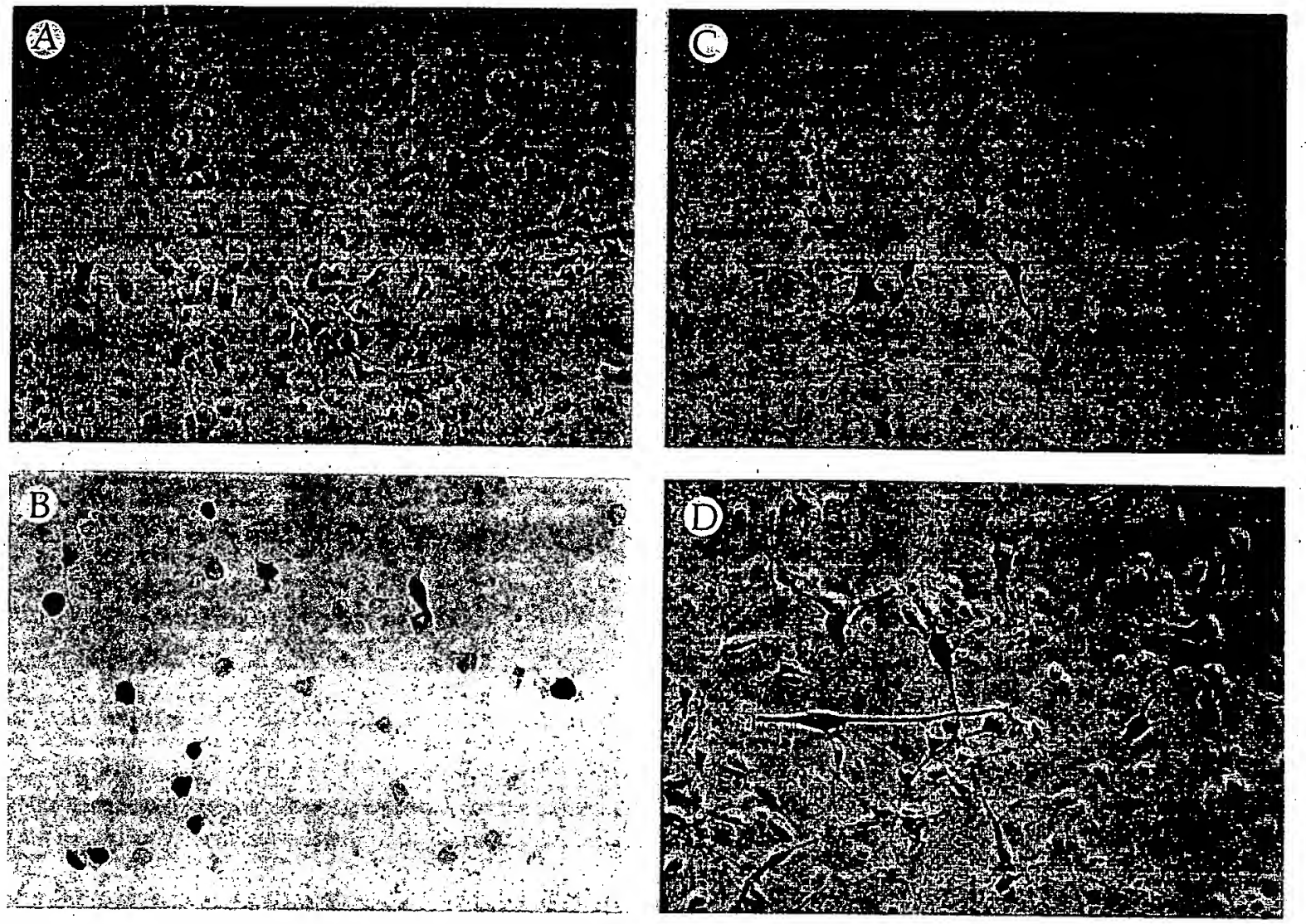
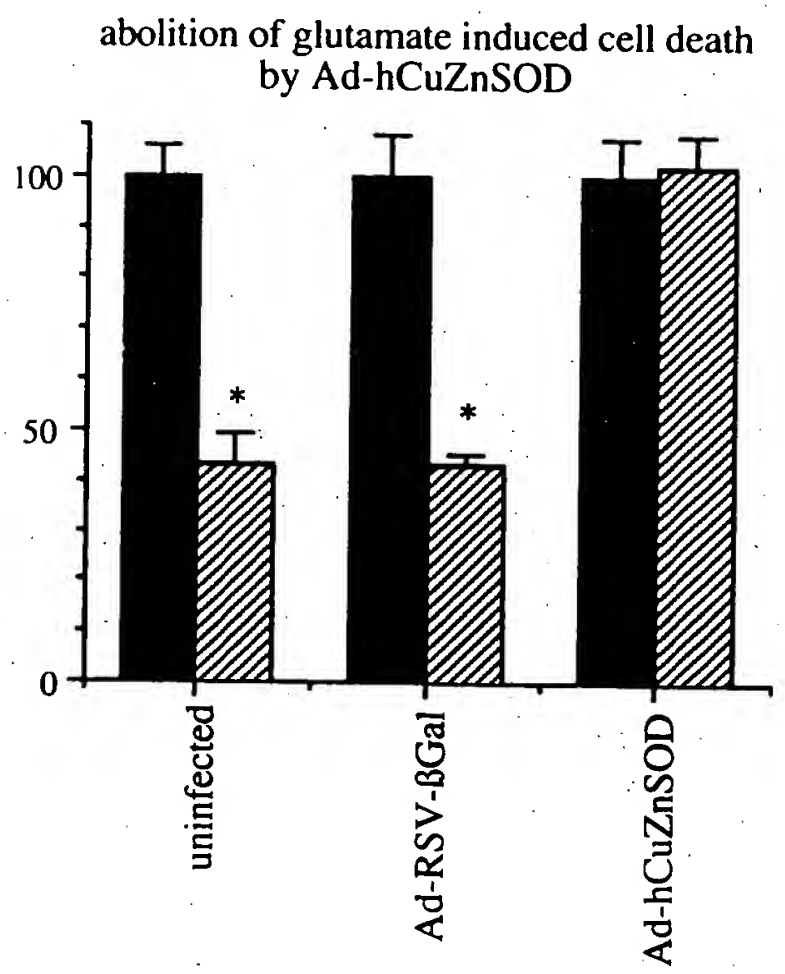


FIG. 3. Expression of transgenes in striatal neurones *in vitro*. Striatal cells were infected with Ad-βGal or Ad-hCuZnSOD at a MOI of 300 pfu cell^{-1} after 4 days *in vitro* and cells were fixed 3 days later to test transgene expression. (A) Uninfected cells (×20), (B) Ad-βGal-infected cells (×40), (C-D) Ad-hCuZnSOD infected cells (respectively ×20 and ×40).



and 300 pfu cell^{-1}. The expression of β-galactosidase or hCuZnSOD in striatal cells was detected by X-Gal staining or hCuZnSOD immunochemistry respectively (Fig. 3). No β-galactosidase activity was detected in Ad-hCuZnSOD-infected or control cells, and no hCuZnSOD was detected in Ad-βGal-infected or control cells (data not shown).

High doses of glutamate (2.5 mM for 24 h) applied to control primary cultures of striatum led to the death of 55% of cells, as evidenced by a decrease in fluorescein diacetate staining and a parallel increase in propidium iodide staining (Fig. 4). Infection of striatal cells with Ad-hCuZnSOD (100 pfu.cell^{-1}) conferred protection against glutamate-induced toxicity. No mortality following glutamate application was ob-

FIG. 4. Striatal neurones were infected with Ad-βGal or Ad-hCuZnSOD at a MOI of 100 pfu cell^{-1} after 4 days *in vitro*. Three days after infection, cells were exposed (▨) or not exposed (■) to 2.5 mM glutamate for 24 h and stained for β-galactosidase or hCuZnSOD. βGal and hCu2nSOD expressing cells were counted in 15 microscope fields for each well, and results were expressed as a percentage of the control value (i.e. the number of βGal and hCu2nSOD positive cells in cultures not subjected to glutamate toxicity ± s.e.m.). Triplicate sister cultures were subjected to each condition and the experiment was repeated three times. *$P<0.002$ (student's *t*-test). Cell survival in control cultures (uninfected cells) was determined by the FDA/PI method.

served among Ad-hCuZnSOD-infected cells, as demonstrated by counting cells immunoreactive for hCuZnSOD in glutamate-treated and untreated cultures (Fig. 4). This protective effect was due to the CuZnSOD transgene and not to viral infection *per se*: 55% of the βGal positive cells (AdβGal infected cells) died after glutamate treatment, a mortality rate indentical to that for uninfected cells in control cultures. Thus adenoviral-mediated overexpression of the hCuZnSOD gene protects striatal neurones in primary culture against glutamate-induced toxicity.

Discussion

Studies demonstrating the reduced vulnerability of hCuZnSOD transgenic mice to numerous toxic insults have indicated the neuroprotective potential of CuZnSOD. However, the use of such an enzyme to prevent neuronal death is hampered by its inability to cross cell membranes. For instance, NMDA-dependent superoxide production *in vitro* leads to neurotoxicity, and this cell death cannot be blocked by extracellular SOD.[16] Similarly, in an *in vitro* model of hypoxia (in which glutamic acid is implicated as the proximal cause of neurodegeneration), superoxide dismutase protects neurones only when taken up intracellularly under depolarizing conditions.[17] Finally, CuZnSOD delays apoptosis of sympathetic neurones deprived of growth factor, but only when the enzyme is microinjected into cells, and not when it is added to the extracellular medium.[7] These results demonstrate that CuZnSOD has to accumulate intracellularly to be able to protect injured cells. Thus, an appropriate vector is required to accomplish efficient CuZnSOD delivery to neurones. We show that adenoviral-mediated gene transfer is an efficient way to produce hCuZnSOD in neuronal cells. The exogenous enzyme is functional, as shown by activity tests in non-denaturating polyacrylamide gel. Furthermore, the intracellular levels of CuZnSOD are sufficient to protect neurones from glutamate-induced cell death.

Glutamate neurotoxicity can be mediated by the activation of glutamate receptors, or by the inhibition of cystine uptake (through the cystine/glutamate antiporter system): both mechanisms lead to the formation of free radicals. The stimulation of both ionotropic and metabotropic receptors is known to induce a rise in the cytosolic concentration of calcium which may enhance the production of free radicals by the activation of (1) a phospholipase yielding superoxide by the release and subsequent metabolism of arachidonic acid and (2) a protease which in turn accelerates the conversion of xanthine deshydrogenase to xanthine oxidase, a cellular source of superoxide.[18] Moreover, the activation of glutamate receptors may also trigger nitric oxide (NO) production,[19] yielding the peroxynitrite radical through its reaction with superoxide. Glutamate toxicity involving inhibition of cystine uptake, leading to glutathione depletion and free radical generation, has also been described in neuronal cell lines[20,21] and in cells in primary culture[22].

Independent of the possible mechanisms of glutamate toxicity, the glutamate-mediated production of free radicals is prevented in striatal cells infected by Ad-hCuZnSOD. This observation has two implications. First it suggests that in the striatal neurone model we used, superoxide radicals are involved in glutamate neurotoxicity, as they are in cerebellar[16] and cortical cells.[6] Second, our data show that Ad-hCuZnSOD is a potential therapeutic tool to prevent neurodegeneration associated with glutamate neurotoxicity. Interestingly, a recent report published during the preparation of this paper showed that Ad-hCuZnSOD could protect sympathetic neurones against NGF deprivation-induced death *in vitro*.[23] Thus Ad-hCuZnSOD displays a neuroprotective effect against both glutamate toxicity and growth factor-dependent neuronal death. Adenovirus-mediated hCuZnSOD gene transfer may therefore have therapeutic applications for a wide range of neurodegenerative disorders.

Conclusion

A high dose of glutamate applied to striatal neurones in primary culture leads to a cell death rate of 55% due to glutamate-induced oxidative stress. A recombinant adenovirus encoding hCuZnSOD is able to direct the production of this enzyme in striatal cells, and thereby to protect them against glutamate toxicity by detoxifying free radicals.

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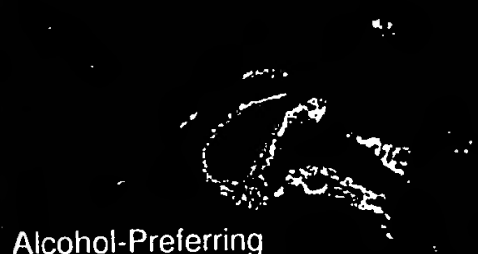

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ADENOVIRUS IN THE BRAIN: RECENT ADVANCES OF GENE THERAPY FOR NEURODEGENERATIVE DISEASES

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Abstract—Adenovirus is an efficient vector for neuronal gene therapy due to its ability to infect post-mitotic cells, its high efficacy of cell transduction and its low pathogenicity. Recombinant adenoviruses encoding for therapeutical agents can be delivered *in vivo* after direct intracerebral injection into specific brain areas. They can be transported in a retrograde manner from the injection site to the projection cell bodies offering promising applications for the specific targeting of selected neuronal populations not easily accessible by direct injection, such as the motor neurons in the spinal cord. Adenoviral vectors are also efficient tools for the *ex vivo* gene therapy, that is, the genetical modification of cells prior to their transplantation into the nervous system. Recently, the efficacy of the adenovirus as a gene vector system has been demonstrated in several models of neurodegenerative diseases including Parkinson's disease (PD) and motor neuron diseases. In rat models of PD, adenoviruses encoding for either tyrosine hydroxylase, superoxide dismutase or glial-derived neurotrophic factor improved the survival and the functional efficacy of dopaminergic cells. Similarly, the intramuscular injection of an adenovirus encoding for neurotrophin-3 had substantial therapeutic effects in a mutant mouse model of motor neuron degenerative disease. However, although adenoviruses are highly attractive for neuronal gene transfer, they can trigger a strong inflammatory reaction leading in particular to the destruction of infected cells. The recent development of new generations of adenoviral vectors could shed light on the nature of the immune reaction caused by adenoviral vectors in the brain. The use of these new vectors, combined with that of neurospecific and regulatable promoters, should improve adenovirus gene transfer into the central nervous system.

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1. INTRODUCTION

Human gene therapy is the term that refers to the introduction into somatic cells of new genetic material of therapeutic value. This innovative approach is potentially a powerful method for treatment of neurological diseases for which classical pharmacotherapy is unavailable or not easily applicable. Due to the presence of the blood brain barrier (BBB), the central nervous system (CNS) is not easily accessible to systemically delivered macromolecules with therapeutic activity such as growth factors, cytokines or enzymes. These macromplecules must be directly injected *in situ* to reach the brain parenchyma. In addition, the limited half-life of these potentially therapeutic proteins requires repeated intracerebral injections or infusions using osmotic pumps. The technical complexity of these procedures, and the increased risks of infection associated with repeated BBB disruptions, hinder the clinical application of recent advances in our knowledge of the pathogenesis of nervous diseases. Intracerebral grafting of embryonic brain cells has long been proposed as an alternative to the pharmacological substitution of a deficient neurotransmitter (Bjorklund and Stenevi, 1979). However, this palliative approach is hampered by ethical considerations associated with the use of human fetal tissue, in addition to problems of acquisition of fetuses: numbers are needed to obtain sufficient material for grafting a single patient. The grafting procedure also requires immunosuppression, with its major impact on the subsequent quality of life of the patients. Finally, the symptomatic relief obtained after neuronal transplantation in patients with PD is not complete, possibly being due to the relatively poor survival of the implanted neurons. Gene therapy may allow these problems to be overcome by locally producing the desired therapeutic protein in a given brain structure and making it available for prolonged periods after a single administration.

The first issue for developing an efficient gene therapy for the nervous system is the identification of potentially beneficial agents and the subsequent cloning of their genes. Promising candidates for gene therapy in the nervous system are the neurotrophic factors. For example, the glial cell line-derived neurotrophic factor (GDNF) and the nerve growth factor (NGF), considerably improve the survival of motor and dopaminergic neurons (Beck *et al.*, 1995; Oppenheim *et al.*, 1995), and that of cholinergic neurons (Fischer *et al.*, 1987), respectively. Antioxidative agents, particularly oxidative metabolism enzymes such as superoxide dismutase, catalase or glutathione peroxidase, are also theoretically of major interest for neuroprotection in all pathological conditions involving free radicals (amyotrophic lateral sclerosis, Parkinson's, Alzheimer's and Huntington's diseases, cerebral ischemia and others) (Olanow, 1993). The development of an antioxidant-based gene therapy is particularly promising as antioxidative compounds are generally without toxicity, and they potentially have a non-specific protective effect on each neuron undergoing neurodegenerative process.

The choice of an appropriate vector system for transferring the desired gene into the affected brain area is the second issue for efficient gene therapy. Two strategies can be adopted: genes can be transferred either directly by intracerebral injection of a gene vector system (*in vivo* gene transfer) or indirectly by grafting genetically modified cells into the brain (*ex vivo* gene transfer). Currently, the most efficient method for both these strategies is based on the use of modified viruses as biological vector systems, because of their naturally propensity to transfer their own genome into the cells that they infect. Three types of virus are most widely used to carry therapeutic genes: the retrovirus, the adenovirus, and the adeno-associated virus. We believe that the adenovirus is currently one of the most appropriate vectors for neuronal gene therapy because of its ability to infect post-mitotic cells, its high efficiency of cell transduction, and its low pathogenicity.

This review considers the potential of recombinant adenoviruses in the development of efficient gene therapy for disorders of the nervous system, and in particular for neurodegenerative diseases.

2. ADENOVIRUSES ARE EFFICIENT GENE VECTORS FOR *IN VIVO* GENE THERAPY

2.1. Direct Injection

First generation recombinant adenoviruses are currently constructed by substitution of the viral E1/E3 gene regions with therapeutic genes, and amplification in a *trans*-complementing cell line established from human embryonic kidney cells, designed 293. These replication-defective adenoviruses can infect a wide range of cell types, including post-mitotic cells. The remarkable efficiency of adenoviral vectors for introducing foreign genetic material into rodent nervous cells *in vitro* and *in vivo* was first demonstrated by Le Gal La Salle *et al.* (1993). They used a recombinant replication-defective adenovirus encoding for the *Escherichia coli lacZ* reporter gene under the control of a strong virus promoter (Ad-RSVβgal) to infect primary culture cells from superior cervical ganglia. They demonstrated expression of β-galactosidase (βgal) in almost all cultured cells (sympathetic neurons and astrocytes). Furthermore, stereotactic intracererebral inoculation of the Ad-RSVβgal into hippocampus and substantia nigra allowed the extensive labeling of neurons, astrocytes and microglial cells around the injection site. The intracellular expression of the marker gene was detected for about 2 months, with only minimal neuropathological consequences. This ability of adenoviruses to infect neuronal cells *in vivo* was described at the same time by several other groups, who confirmed the relatively long-term expression of the transgenes, and the limited toxicity of moderate titer of virus inoculum (Davidson *et al.*, 1993; Akli *et al.*, 1993; Bajocchi *et al.*, 1993).

2.2. Retrograde Transport

Interestingly, adenovirus vectors can be transported in a retrograde manner from the injection

site to the projection cell bodies, following uptake at nerve terminals (Akli *et al.*, 1993; Ridoux *et al.*, 1994a). By double-labeling nigral cells histochemically and immunochemically for β-galactosidase and tyrosine-hydroxylase, Ridoux *et al.* (1994a) provided evidence of Ad-RSVβgal uptake at the injection site in rat striatum and retrograde transport to the ipsilateral substantia nigra. These results were followed by those of Kuo *et al.* (1995) who further documented only minor uptake of the vector by fibers after injection into the genu of the corpus callosum, suggesting that uptake by fibers-of-passage should not be a problem in tracing studies using adenovirus vectors within the central nervous system (CNS) (Kuo *et al.*, 1995). But the most interesting application of this ability of adenovirus to be retrogradely transported is that it could be used for the specific targeting of selected neuronal populations not easily accessible by direct injection, while avoiding any undesirable side effects associated with systemic administration (Finiels *et al.*, 1995) or tissue damage due to viral toxicity at the site of injection (Cayouette and Gravel, 1996). Retrograde axonal transport of adenoviruses is particularly applicable to gene therapy for fatal neurodegenerative diseases of motor neurons, including amyotrophic lateral sclerosis, Kennedy's disease and spinal muscular atrophy. Indeed, following injection into muscles, recombinant adenoviruses can be retrogradely transported through the axons of motor neurons, from neuromuscular junctions to motor neuron cell bodies (Ghadge *et al.*, 1995; Finiels *et al.*, 1995). Finiels *et al.* found that this simple method of gene transfer allowed specific and high-level transgene expression (β-galactosidase) in the rat spinal cord, with 50–100% of the afferent motor neurons being infected after injection of 2.7×10^8 plaque forming units (pfu) per muscle. The β-galactosidase expression persisted, albeit with progressive loss, for at least 30 days post-injection (Finiels *et al.*, 1995). In addition, Ghadge *et al.* (1995) described the infection of hypoglossal cranial nerve nuclei, as well as sensory neurons and tracts, following inoculation of the *lacZ* adenovirus into the mouse tongue. No sign of inflammation was reported in the CNS in the first 10 days after inoculation, and thus may have been due to the presence of less viral protein in neurons than in the inoculated muscle.

2.3. Global Delivery

Bajocchi *et al.* (1993) demonstrated that it was possible to direct the secretion of α1antitrypsin (αAT) into the cerebrospinal fluid by delivering the α1AT gene to ependymal cells after administration of the recombinant α1AT adenovirus to the lateral ventricules of rats. Injection of adenovirus into cerebrospinal fluid of rats (via cisterna magna) further allowed the transduction of cerebral blood vessels *in vivo* [leptomeningeal cells, adventitial and smooth muscle cells; Ooboshi *et al.* (1995)]. The targeting of adenoviral vectors to ependymal cells may thus be a means for a widespread delivery of the therapeutic factor, while avoiding the tissue damage possible following direct intraparenchymal injections.

Other routes for global administration of adenoviruses into the brain have been described in rats including nasal instillation (Draghia *et al.*, 1995) or injection into the carotid artery after BBB disruption with hyperosmotic mannitol (Doran *et al.*, 1995). In the latter study, intracarotid injection of the adenovirus resulted in the expression of the marker gene (*lacZ*) in the pericapillary astrocytes of the ipsilateral cerebral cortex and deep gray matter, and the level of expression correlated with the degree of barrier opening (Doran *et al.*, 1995). Muldoon *et al.* (1995) also used mannitol-mediated disruption of the BBB for intracerebral delivery of adenoviruses. In their study, disruption of the BBB considerably increased the area of distribution of the virus, with transgene expression detected throughout the disrupted cerebral cortex. The authors added that most infected cells were of glial morphology, which makes this route of virus administration particularly promising for global delivery of secreted therapeutic factors to the CNS (Muldoon *et al.*, 1995).

2.4. *Ex Vivo* Strategy

Studies by Ridoux *et al.* (1994b) indicate that adenoviral vectors are also efficient tools for *ex vivo* gene therapy, that is the genetical modification of cells before their transplantation into the CNS. In this study, rat primary astrocytes that were transduced with Ad-RSVβgal and subsequently grafted into various brain structures showed a robust survival and expressed the transgene for at least 5 months. A similar *ex vivo* strategy has been developed using human neural progenitors explanted from germinative zones of the CNS from 6 to 10 week-old human fetuses and amplified in serum-free culture medium containing basic fibroblast growth factor (Buc-Caron, 1995; Sabaté *et al.*, 1995). Amplification *in vitro* of these neural progenitors may be particularly valuable as a way of minimizing the ethical and supply problems raised by the clinical use of human fetal tissue. Large numbers of cultivated progenitors were infected with the Ad-RSVβgal adenovirus (at a multiplicity of infection of 500) and transplanted into the striatum of immunosuppressed rats. Two to three weeks after transplantation, the authors observed a small percentage of surviving neuroblasts strongly expressing β-galactosidase in four out of 13 rats (Sabaté *et al.*, 1995). The combined use of human neural progenitors amplified *in vitro* and recombinant adenoviruses has exciting possibilities for the treatment of neurodegenerative diseases by *ex vivo* restorative strategies.

3. GENE THERAPY FOR NEURODEGENERATIVE DISEASES

3.1. Parkinson's Disease

Parkinson's disease is characterized by the progressive loss of dopaminergic neurons from the substantia nigra, a brain structure which innervates the striatum, leading to specific motor impairment (tremor, rigidity and akinesia) often associated with cognitive troubles. Classic oral administration of L-

Dopa can significantly improve motor function during the first few years of treatment. But, as the neurodegeneration progresses, this therapy becomes less effective requiring higher doses of L-Dopa, which leads to deleterious side-effects. Other therapeutic strategies have thus been proposed including cell grafting as source of dopamine (DA) production in the denervated striatum, or prevention and neuroprotection of the degenerating DA cells by supplying neurotrophic factors to the brain.

3.1.1. *Ex Vivo Restorative Approach Using An Adenovirus Encoding for Superoxide Dismutase*

Transplantation of human fetal dopaminergic cells into patients with PD has given encouraging results (Lindvall *et al.*, 1990). However, the symptomatic relief is not complete, possibly because of the poor survival of the implanted neurons (5–20% of the grafted DA neurons survive the transplantation procedure). Genetic engineering of DA fetal cells with a neuroprotective factor before their grafting into the brain is an attractive strategy for which adenovirus vectors may be particularly valuable. Because they can generate free radicals by auto-oxidation or via the monoamine oxidase catabolism of DA, dopaminergic neurons appear to be particularly exposed to oxidative stress (Olanow, 1992). There may also be additional oxidative stress during preparation and implantation of the mesencephalic tissue, due to cellular hypoxia and trauma (Nakao *et al.*, 1994). Furthermore, the survival rate of ventral mesencephalon (VM) transplants from transgenic mice for the human copper–zinc superoxide dismutase (hSOD-1), an antioxidative enzyme, was much higher than that of transplants from non-transgenic littermates (Nakao *et al.*, 1995). A clinical application of this finding could be to use an adenovirus vector encoding hSOD-1 for infecting VM tissue before grafting it into the denervated striatum. We recently constructed a replication-defective adenovirus containing the hSOD-1 cDNA downstream from the Rous Sarcoma Virus (RSV) promoter. It efficiently directed the intracellular expression of hSOD-1, and protected neuronal cells in culture from glutamate-mediated oxidative stress (Barkats *et al.*, 1996). We used this recombinant adenovirus for transducing cell suspensions of rat VM, and then transplanted the cells into the denervated striatum of adult rats with unilateral 6-hydroxydopamine (6-OHDA) lesion of the mesostriatal pathway (Barkats *et al.*, 1997). This lesion causes depletion of DA in the denervated striatum, inducing a turning behavior in response either to pharmacological stimulation of striatal hypersensitive DA receptors or to increasing the striatal release of DA (Ungerstedt and Arbuthnott, 1970). Control cell suspensions were either not infected, or infected with an adenovirus bearing the *lacZ* marker gene under the control of the RSV promoter. Human SOD-1 and β-galactosidase were produced in the grafts four days after transplantation, and the expression persisted at 5 weeks thereafter, as revealed by immunohistochemistry. Five weeks post-grafting, there was a more extensive functional recovery in the SOD group than in the control groups. Interestingly, the mean number of surviving DA cells in the SOD grafts was nearly double that in controls (443 ± 129, 347 ± 130 and 700 ± 150 for the non-infected, βgal and SOD groups, respectively), although this difference was not statistically significant. The inflammatory consequences of the adenovirus gene transfer were minimal, and only a moderate microglial response around the graft tissue was detected by immunohistochemical analysis of complement receptor 3 (CR3). The potential of adenovirus for *ex vivo* gene transfer is clear from comparison of these and previous findings obtained when comparing our findings to previous ones obtained using a defective herpes simplex virus (HSV-1) vector for the *ex vivo* transfer of the *lacZ* and the TH genes into the rat brain (Sabel *et al.*, 1995). Indeed, in this study, the authors did not find a single TH-expressing cell in the grafts and only a few *lacZ*-positive cells, due to the toxicity of the HSV-1 gene transfer vector.

Thus, the replication-defective, hSOD-1 adenovirus could be an efficient tool for enhancing survival and functional efficacy of neuronal, or non-neuronal, grafted cells. Further studies directed towards the obtainment of a higher level of transgene expression, combined with a minimal inflammatory reaction should improve the efficacy of this recombinant adenovirus for transplantation purposes.

3.1.2. *In Vivo Restorative Approach Using An Adenovirus Encoding for Tyrosine Hydroxylase*

Another study using the adenovirus as a gene vector for PD gene therapy shows that intrastriatal transduction of the tyrosine hydroxylase (TH) gene can lead to a significant behavioral recovery of 6-OHDA lesioned rats (Horellou *et al.*, 1994). An adenovirus carrying the human TH-1 cDNA under the control of the RSV promoter was directly inoculated into the brain, 3–4 weeks after the 6-OHDA lesion. An optimal dose of 15×10^7 particles was stereotactically injected into nine sites in the striatum at a low delivery rate to promote the diffusion of the virus and dispersion of transgene expression, but minimize tissue damage. Infected striatal cells produced DA, resulting in a reduction of DA receptor hypersensitivity which significantly decreased apomorphine-induced turning 1 and 2 weeks after injection of Ad-RSVhTH. TH production in infected tissues was detected immunohistochemically. Most infected cells were reactive astrocytes, although some TH-immunoreactive neurons were also detected. The behavioral improvement observed after the AdRSVhTH injection may therefore have been due to the production of Dopa by the infected astrocytes (Lundberg *et al.*, 1996), and subsequent decarboxylation of the Dopa in the surrounding host striatum (Horellou *et al.*, 1990).

Similar findings of TH compensation in lesioned rats have been obtained using HSV-1 and adeno-associated vectors (AAV) as vector systems for TH gene transfer into the rat striatum (During *et al.*, 1994: Kaplitt *et al.*, 1994). However, these viral vector systems require further safety improvements before clinical application for neurological diseases,

in view of the risk of contamination by competent viruses.

3.1.3. *In Vivo Protective Approach Using An Adenovirus Encoding for GDNF*

A different strategy is to protect DA nigral cells from the neurodegenerative process by engineering them genetically to produce a neurotrophic factor. The feasibility of this approach has recently been demonstrated using a recombinant adenovirus vector (Choi-Lundberg *et al.*, 1997; Bilang-Bleuel *et al.*, 1997). An adenovirus encoding the human GDNF (Ad-GDNF) was found to protect dopaminergic neurons both in culture and in a rat model of progressive neurodegeneration (Bilang-Bleuel *et al.*, 1997). Astrocytes and mesencephalic cells in culture infected with Ad-GDNF secreted 54-fold and 12-fold more trophic factor than control cultures, respectively. Furthermore, when embryonic DA neurons were co-cultivated with Ad-GDNF infected astrocytes, their survival was better than that of control co-cultures (two-fold higher suvival rate). The DA neurons were also clearly more differentiated, had larger cell bodies and more branched processes.

The adenovirus encoding for GDNF was then tested *in vivo* in a model of progressive DA cell degeneration subsequent to the unilateral injection of 6-OHDA into the striatum (Sauer and Oertel, 1994). The GDNF adenovirus was injected into the striatum 6 days prior to the 6-OHDA lesion, allowing the production of GDNF at both DA nerve terminals and nigral cell bodies via retrograde transport of the virus. Three weeks after the lesion, morphometrical analysis of the substantia nigra revealed twice as many TH-immunoreactive cell bodies in the Ad-GDNF treated animals than in control animals (about 60% vs 30% of TH-positive neurons). Furthermore, the amphetamine-induced turning (behavioral marker for DA depletion) was less pronounced in the GDNF group than in control group 1–3 weeks after the lesion.

A similar neuroprotective effect of an adenovirus encoding GDNF has also been reported by Choi-Lundberg *et al.* (1997) in the 6-OHDA progressive lesion rat model of PD, although they did not use the same method of morphometrical analysis nor the same protocol of adenovirus injection. A subpopulation of DA cells were retrogradely labeled with bilateral injections of fluorogold into the striatum prior to the unilateral injection of GDNF and control adenoviruses dorsal to the substantia nigra. Seven days later, the neurotoxin was injected into the striatum. Morphometrical analysis of the substantia nigra 6 weeks after the lesion revealed nearly 80% survival of the fluorogold-positive DA-like neurons in AdGDNF-treated animals, compared to 30% survival in controls. However, this study does not document the protective effect of Ad-GDNF on animal behavior. These two studies demonstrate that an increased level of GDNF resulting from the expression of an adenovirus vector injected either in the striatum or near the substantia nigra can protect DA neurons from degeneration caused by exposure of their terminals to 6-OHDA. The study of Bilang-Bleuel and collaborators shows that in addition to the DA cell bodies, DA innervation of the striatum is also preserved, possibly accounting for the observed behavioral effect.

These findings open the way for new treatments for PD based on adenovirus gene transfer technology.

3.2. Motor Neuron Degenerative Diseases

3.2.1. *Therapeutic Effect of An Adenovirus Encoding for Neurotrophin-3*

Neurotrophic factors are potential candidates for treating degenerative diseases of motor neurons for which there is virtually no therapy currently available (Thoenen *et al.*, 1993). Results of several phase II/III clinical trials have shown that systemically administered neurotrophic factors including ciliary neurotrophic factor (CNTF), insulin-like growth factor (IGF-I) and brain-derived neurotrophic factor (BDNF), have no large beneficial effect in patients with amyotrophic lateral sclerosis (ALS). This may be due to limited access of these compounds to motor neurons, their potential deleterious side-effects, and in the case of IGF-I, by the ubiquitous distribution of binding proteins and receptors. Also, a possible down-regulation of BDNF binding sites has been described after repeated administration of this factor at high doses. The continuous delivery of physiological amounts of neurotrophic factors, possible by gene tranfer technology, may help avoid these problems. Indeed, this approach has recently been successful in the mouse progressive motor neuronopathy mutant (*pmn*) using an adenovirus as a vector for gene transfer (Haase *et al.*, 1997). The study demonstrated that an intramuscular injection of an adenovirus vector containing neurotrophin-3 (NT-3) had substantial therapeutic effects in the mutant mouse. The unilateral injection of the NT-3 recombinant adenovirus (1×10^9 pfu) into three muscle groups of neonatal *pmn* mice led to a 100-fold increase in NT-3 production in the injected muscles, without immediate or delayed toxicity. The increase of NT-3 expression was associated with an improvement of the mean life-span: about 61 days for treated *pmn* mice and about 40 days for *pmn* control mice. Electromyographical analysis was used to evaluate the effect of the treatment on neuromuscular function. The amplitude of the evoked motor response was recorded in the gastrocnemius after stimulation of the sciatic nerves. In the AdNT-3-treated mice, the amplitude was 70% of the value in normal mice compared to 40% of normal for untreated *pmn* mice. The values were similar for right and left hindlimbs of unilaterally injected mice, which is compatible with systemic release of NT-3 from the injected muscle and not retrograde transport of the Ad-NT3 to motor neuron cell bodies from the injected muscles. Electromyographical data from the diaphragm (analysis of the motor unit potentials during spontaneous contraction) revealed an increase in the size of the motor units in AdNT-3 treated mice. This finding suggests that NT-3 stimulated the sprouting of motor neurons that did not innervate injected

muscles. This effect of AdNT-3, at distance from the injection site, further argues for a production of the neurotrophic factor in the infected muscle cells, followed by its secretion into the circulation.

The effect of treatment with Ad-NT3 was histopathologically assessed on the phrenic nerves, which innervate respiratory muscles including the diaphragm. In treated *pmn* mice, the loss of myelinated fibers in the phrenic nerves was 30% lower than that in untreated and in Ad*lacZ*-treated *pmn* mice. The effect on axon survival of a combined treatment with Ad-NT-3 and a recombinant adenovirus coding for CNTF was still more pronounced than those observed using each adenoviral vector alone.

In *pmn* mice, motor neuron degeneration starts at the muscle endplates, and retrograde degeneration leads to the loss of motor neurons in the late stages of the disease. Presumably, NT-3 and CNTF, delivered to neonatal *pmn* mice, promote distal axon maintenance and regeneration. The peripheral administration of adenoviruses encoding NT-3 and CNTF may thus be useful for the maintenance and the sprouting of nerve terminals, and for improving motor function in patients suffering from neuromuscular diseases. Transgenic mice expressing a human superoxide dismutase mutation (Gurney *et al.*, 1994) may be a suitable animal model for testing the effect on motor neuron degeneration of neurotrophic or neuroprotective factors encoded by adenoviral vectors. In these transgenic mice, the expression of high levels of human SOD containing a glycine to alanine substitution at position 93 (a mutation which is found in patients with familial ALS) causes motor neuron disease and death at 5–6 months of age. As oxidative stress is probably involved in the neurodegenerative process, treatment with adenovirus vectors encoding antioxidative enzymes may be beneficial in the mutated SOD transgenic mice, and in patients with ALS. To investigate the exact role of NT-3 in motor neuron degeneration, and its potential for promoting motor neuron survival, it would be interesting to examine the effect of intramuscular injections of NT-3/CNTF adenoviruses, alone or in combination, in this transgenic mouse model.

3.2.2. *Prevention Against Axotomy-Induced Cell Death Using Adenoviruses Encoding for GDNF, BDNF or CNTF*

Recently, adenoviruses have also been successfully used to introduce neurotrophic factors into facial motor neurons of newborn rats, and rescue them from degeneration following axotomy (Giménez y Ribotta *et al.*, 1997; Gravel *et al.*, 1997). Giménez y Ribotta *et al.* investigated the protective effect of injecting GDNF and BDNF recombinant adenoviruses into the nasolabial and lower lip muscles of newborn rats on the death of the axotomized facial neurons. The adenoviruses were retrogradely transported to the motor neuron cell bodies before axotomy, to prevent the degenerative process. There was significantly better survival of axotomized motor neurons one week after surgery in rats pretreated with Ad-BDNF (34.5%) or Ad-GDNF (41.2%) than with Adβgal. Similarly, in the study reported by Gravel *et al.* (1997), a significant number of axotomized motor neurons were rescued when rats were injected with adenoviruses encoding CNTF (Ad-CNTF) or BDNF (Ad-BDNF), but not when rats were injected with a *lacZ*-expressing adenovirus. Note that the adenoviruses were injected into the proximal nerve stump immediately after the nerve lesion. The survival effect was observed 1 week after lesion and injection of the viruses, and persisted, although diminished, for at least at 5 weeks. This long-term effect differs from the transient rescue obtained by applying recombinant neurotrophic factors to axotomized neurons which lasts only 2 weeks (Vejsada *et al.*, 1995).

These innovative studies demonstrate the potential of recombinant adenoviruses for the efficient long-term protection of motor neurons. This technology thus holds out hope for the treatment of severe degenerative diseases such as ALS, for which no classical treatment has proven good efficacy.

3.3. CNS Aging and Alzheimer's Disease

Numerous studies have proven that the regulation and survival of the basal forebrain cholinergic neurons are dependent on the presence of neurotrophic factors (Tuszynski and Gage, 1994). The intracerebroventricular infusion of the NGF can further prevent cholinergic neuronal atrophy which is observed in aged rats, and the associated memory impairments (Fischer *et al.*, 1987). Nevertheless, treated animals suffer adverse side-effects, including severe weight loss, and nonspecific sprouting of nerve fibers from various origins. In order to palliate these drawbacks, the trophic effect on the basal forebrain cholinergic neurons of aged rats of injection of NGF-encoding adenovirus (Ad-NGF) into the nucleus basalis magnocellularis (NBM) has been thus investigated (Castel-Barthe *et al.*, 1996). As expected, no weight loss was observed in the AdNGF-injected rats, probably due to the targeted action of the growth factor on the injected structures. Interestingly, there was a significant ipsilateral increase of the cholinergic cell soma areas 3 weeks after the unilateral NBM injection of Ad-NGF (10^6 pfu), but not after injection of the control *lacZ* adenovirus. This suggests that recombinant adenoviruses directing the expression of growth factors can protect neurons against the age-related degenerative process.

The adenovirus gene transfer technology has not still proven its efficacy for treating Alzheimer's disease (AD), as no functional recovery has been demonstrated in experimental animals. The absence of a suitable animal model for AD, combined with the fact that this pathology is characterized by a diffuse degeneration of diverse neuronal cell populations, does not make efficient human gene therapy easy. However, the adenovirus-mediated transfer of genes coding for secreted agents which protect neurons from the degenerative process may be of value in the treatment of AD. Oxidative stress has been widely implicated in amyloid-mediated toxicity (Behl *et al.*, 1994; Bruce *et al.*, 1996), which is believed to be linked to neurodegeneration in AD. Adenoviruses encoding antioxidative enzymes could

therefore be promising candidates for inhibiting the degenerative process associated to AD.

4. LONGEVITY, REGULATION AND TARGETING OF TRANSGENE EXPRESSION

Although adenovirus vectors are highly attractive for gene transfer into nervous cells *in vivo*, there are three major drawbacks:

1. the immune and inflammatory response to the virus;
2. the instability of transgene expression;
3. the possible harmful effects of the delivered factors on non-targeted cells.

Injection of first generation adenoviruses (e.g. adenoviruses that lack E1 and E3 sequences) into immunocompetent humans or animals can cause substantial immune reaction (Yang *et al.*, 1994a,b). The adenoviral infection can trigger a strong inflammatory reaction affecting the patient's health, and also leading to the shut down of transgene expression as a consequence of the destruction of host cells. The immunogenicity of the first generation recombinant adenoviruses may be results of toxic reactions triggered by early (DNA binding protein) and late (penton, hexon, fiber) viral proteins (Michou *et al.*, 1997), or by the transgene itself (Tripathy *et al.*, 1996). The inflammation caused by the virus involves both an early innate non-specific response, and a later adaptative immune response. During this later phase, the exogenous proteins are processed in the cytoplasm and presented on the cell surface, allowing the recognition and subsequent cell destruction by cytotoxic T cells (CTL). When adenoviruses are injected into the brain, long-term expression of the transgene is possible in spite of the ensuing inflammatory response (Byrnes *et al.*, 1996). This persistent expression in the brain may be because the intracerebral injection of adenoviral vectors triggers only an ineffective T cell response. Byrnes *et al.* (1996), suggest that the antigen-specific T cell-mediated immune response might therefore not account for the declining expression from adenoviral vectors. One possibility could thus be that damage caused by the early T cell-independent inflammatory response to the virus is responsible for the decline in expression. However, the mechanism causing transgene expression to decline over time is not completely clear, and the involvement of the T-cells in the inflammatory response to the virus in the brain is still debated.

To try to get around this problem, new generations of adenoviral vectors have been developed, by deleting additional viral genes, such as the E2 (Engelhardt *et al.*, 1994; Yang *et al.*, 1994a,b) or the E4 sequences (Dedieu *et al.*, 1997). Analysis of the properties of these improved adenoviral vectors could shed light on the nature of the immune reaction triggered by adenoviral vectors in the brain. The use of 'gutless' vectors, from which essentially all of the viral genes have been eliminated (except the packaging sequence and the elements that define the beginning and the end of the viral genome) could allow an increased persistence of transgene expression (Chen *et al.*, 1997). Immunosuppressive drugs, such as cyclosporine could also be administered simultaneously with virus infection to reduce the immune reaction. Engelhardt *et al.* (1994) showed that a cyclosporin treatment of mice infected with first generation adenoviruses increases stability of transgene expression. Transient immunosuppressive therapies using antibodies which block interactions between T cells and antigen presenting cells, could also be used to prolong transgene expression as well as to allow secondary vector administration (Kay *et al.*, 1997). However, manipulation of the vector is always preferable to that of patients, especially when they are already weakened by severe disease. Recently, immunosuppressive genes have been incorporated into an adenoviral vector (Qin *et al.*, 1997). The adenovirus-mediated gene transfer of the viral cytokine interleukin-10 into murine cardiac allografts improved the persistence of the vector and extended gene expression of a co-injected *lacZ* adenovirus, by reducing adenovirus-specific CTL response. Such a strategy could be considered for improving adenovirus gene transfer into the CNS.

One of the variables which may also influence the duration of transgene expression is the promoter. The decline in expression over time observed after adenovirus infection may be due to the gradual inactivation of the strong promoter elements from the RSV or the cytomegalovirus (CMV) that are generally used. This known shut-down of transgene expression driven by viral promoters *in vivo* (Palmer *et al.*, 1991) could be prevented by using cellular house-keeping promoters or CNS-specific promoters (which have the added benefit of allowing precise targeting of expression to particular cell types). Several promoters specific for the CNS can be used including the promoter of the neurofilament light chain and that of the neuron-specific enolase for targeting neurons, the glial acidic-fibrillary protein (GFAP) promoter specific for astrocytes, the myelin basic protein promoter specific for oligodendrocytes, the DA β-hydroxylase and the TH promoters for targeting catecholaminergic and dopaminergic cells.

The amount of protein produced from a transgene may also be crucial for efficient gene therapy. Regulatable ('inducible') promoters may thus be appropriate. Recently, a tetracycline (tet)-responsive recombinant adenovirus vector containing a tet repressor/operator-based mammalian gene expression system, has been constructed to control the production of a cytokine (TNF-α) in human tumor cells (Hu *et al.*, 1997). In this system, transgene expression can be negatively regulated by administration of low concentrations of tetracyclines. A similar strategy is being developed in our laboratory to control the expression of transgenes in nerve cells (Corti *et al.*, submitted). In a previous study, Corti *et al.* (1996) showed that the tet-based regulatory system efficiently allows the regulation of luciferase-reporter gene expression in intracerebral grafts of a neural precursor cell line. First generation adenoviruses including the tet-sensitive transactivator and transgenes relevant to CNS gene therapy have been constructed, and experiments designed for testing their ability to drive appropriate levels of transgene expression are currently underway.

5. CONCLUSION

Considerable progress has been made since the first demonstration of the efficacy of adenovirus vectors for gene transfer into nervous cells. In particular, the increased expression of several growth factor genes, introduced into nervous cells using adenoviral vectors, has allowed us to limit cell death or to delay the onset of the clinical signs in experimental models of neurodegenerative diseases. Although further preclinical trials are required to demonstrate the feasability, the efficacy, and the safety of adenoviral gene transfer, the substantial growth in research into experimental gene therapy using adenoviruses is, evidence of the considerable potential of these viral vectors for treatment of neurodegenerative diseases.

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Neuronal transfer of the human Cu/Zn superoxide dismutase gene increases the resistance of dopaminergic neurons to 6-hydroxydopamine

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Abstract

Several mechanisms are thought to be involved in the progressive decline in neurons of the substantia nigra pars compacta (SNpc) that leads to Parkinson's disease (PD). Neurotoxin 6-hydroxydopamine (6-OHDA), which induces parkinsonian symptoms in experimental animals, is thought to be formed endogenously in patients with PD through dopamine (DA) oxidation and may cause dopaminergic cell death via a free radical mechanism. We therefore investigated protection against 6-OHDA by inhibiting oxidative stress using a gene transfer strategy. We overexpressed the antioxidative Cu/Zn-superoxide dismutase (SOD1) enzyme in primary culture dopaminergic cells by infection with an adenovirus carrying the human SOD1 gene (Ad-hSOD1). Survival of the dopaminergic cells exposed to 6-OHDA was 50% higher among the SOD1-producing cells than the cells infected with control adenoviruses. In contrast, no significant increased survival of (6-OHDA)-treated dopaminergic cells was observed when they were infected with an adenovirus expressing the H_2O_2-scavenging glutathione peroxidase (GPx) enzyme. These results underline the major contribution of superoxide in the dopaminergic cell death process induced by 6-OHDA in primary cultures. Overall, this study demonstrates that the survival of the dopaminergic neurons can be highly increased by the adenoviral gene transfer of SOD1. An antioxidant gene transfer strategy using viral vectors expressing SOD1 is therefore potentially beneficial for protecting dopaminergic neurons in PD.

Keywords: adenovirus, dopaminergic neurons, gene therapy, oxidative stress, Parkinson's disease.

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Parkinson's disease (PD) is a neurological disorder characterized by a progressive and massive degeneration of dopaminergic cells in the substantia nigra pars compacta (SNpc). The resulting dopamine (DA) depletion in the striatum leads to the deterioration of motor function which manifests itself by tremor, bradykinesia, and rigidity. There is evidence that oxidative stress contributes to the nigral dopaminergic neuronal degeneration. This stress results from both increased generation of free radicals and impairment of the cell defence system for scavenging them (Dunnett and Björklund 1999). First, the nonenzymatic auto-oxidation of DA or its oxidation by monoamine oxidase-B would generate cytotoxic reactive oxygen species like hydrogen peroxide (H_2O_2) and superoxide (Olanow 1993). Activation of *N*-methyl-D-aspartate (NMDA) receptors by glutamate released from the neocortical or subthalamic inputs to the SN could further increase the generation of toxic free radicals. This process could be enhanced by the high levels of iron

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Abbreviations used: Ad-βgal, adenovirus encoding β-galactosidase; Ad-GPx, recombinant adenovirus encoding SOD1; Ad-SOD1, recombinant adenovirus encoding SOD1; BDNF, brain-derived neurotrophic factor; CMV, cytomegalovirus; DA, dopamine; DAB, diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; HBSS, Hank's balanced salt solution; H_2O_2, hydrogen peroxide; MnTBAP, Mn(III) tetrakis(4-benzoic acid)porphyrin chloride; MOI, multiplicity of infection; NBT, nitroblue tetrazolium; 6-OHDA, 6-hydroxydopamine; PBS, phosphate-buffered saline; PD, Parkinson's disease; PFA, paraformaldehyde; RSV, Rous sarcoma virus; SNpc, substantia nigra *pars compacta*; SOD1, copper-zinc superoxide dismutase; TEMED, tetramethylethylenediamine; TH+, tyrosine hydroxylase immunopositive; X-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactosidase.

associated with low levels of ferritin in the SN of PD patients (Dexter *et al.* 1989, 1990) yielding cytotoxic hydroxyl radicals through the Fenton reaction (Youdim *et al.* 1993). In addition, the SN of PD patients have anormally low levels of reduced glutathione (GSH) (Perry *et al.* 1981) and of antioxidative enzyme activities (Ambani *et al.* 1975; Kish *et al.* 1985; Youdim *et al.* 1993), as well as abnormally high levels of lipid peroxides (Dexter *et al.* 1989).

The 6-hydroxydopamine (6-OHDA)-induced lesion of the nigrostriatal dopaminergic system is commonly used as a model for PD. The neurotoxin is taken up by the dopaminergic neurons leading to the generation of neurotoxic reactive oxygen species resulting from DA auto-oxidation (Kumar *et al.* 1995). 6-OHDA has been detected in the urine of parkinsonian patients (Andrew *et al.* 1993), and it has been suggested to act endogenously in PD (Glinka *et al.* 1997). Injections of the neurotoxin into the rat striatum induce a reduction in the levels of GSH, GSH peroxidase (GPx), and SOD1, together with an increase in lipid peroxidation (Perumal *et al.* 1989; Kumar *et al.* 1995), a situation that mimics some aspects of the oxidative stress associated with PD. Antioxidants such as *N*-acetylcysteine, Mn(III) tetrakis(4-benzoic acid)porphyrin chloride (MnT-BAP), or the C3 carboxyfullerene derivative, protect against 6-OHDA-mediated toxicity in dopaminergic neurons *in vitro* (Choi *et al.* 1999; Lotharius *et al.* 1999). Moreover, the protective potential of the SOD1 antioxidative enzyme against (6-OHDA)-induced toxicity has been demonstrated both *in vitro* using human SY5Y neuroblastoma cells (Tiffany-Castiglioni *et al.* 1982) and *in vivo* with SOD1 transgenic mice. In this study, the mice were protected against the neurotoxic effects of intracerebroventricular injection of the toxin (Asanuma *et al.* 1998). These studies all implicate free radicals in the (6-OHDA)-induced neurotoxicity.

One way to increase the antioxidant potential of dopaminergic cells both *in vitro* and *in vivo* is the transfer of genes encoding antioxidative enzymes into specific cell compartments, such that there is long-term overexpression of the corresponding proteins. This 'antioxidant' strategy of neuroprotection based on gene transfer technology should help increase the resistance of dopaminergic neurons to the oxidative stress generated during PD. We previously described the efficacy of this methodology for increasing survival of neuronal cells in which death was induced by exposure to glutamate or to β-amyloid *in vitro* (Barkats *et al.* 1996, 2000), and to the stress mediated by the intracerebral grafting procedure (Barkats *et al.* 1997). To analyse the protective potential of the DA neuronal gene transfer of SOD1 against (6-OHDA)-induced neurotoxicity, rat embryonic mesencephalic neurons were transduced with an adenovirus vector encoding the human SOD1 enzyme (Ad-SOD1) prior to exposure to 6-OHDA. Active human SOD1 was expressed in the (Ad-SOD1)-infected dopaminergic neurons. These neurons were significantly more resistant to 6-OHDA than either non-infected or control adenovirus-infected neurons. In contrast, the adenovirally mediated overproduction of GPx in dopaminergic neurons did not protect them against the toxic effects of 6-OHDA.

Materials and methods

Recombinant adenoviruses

The human CuZnSOD (Ad-SOD1) and the bovine GPx genes (Ad-GPx) were cloned in an adenoviral backbone under the control of the Rous sarcoma virus (RSV) promoter (Barkats *et al.* 1996, 2000). One control adenovirus, encoding βgalactosidase, expressed the *LacZ* reporter gene under the control of the same promoter (Stratford-Perricaudet *et al.* 1992). The second control adenovirus (empty adenovirus) contained the cytomegalovirus (CMV) promoter without any transgene (Vector Developments, Gencell, Aventis, France). All viral stocks were prepared as previously described (Stratford-Perricaudet *et al.* 1992).

Primary cultures of ventral mesencephalon

We established serum-free ventral mesencephalon cultures enriched for dopaminergic neurons from embryonic day 14 Sprague–Dawley rat midbrain. Tissues were rinsed in phosphate-buffered saline (PBS) containing 0.6% glucose and were incubated at 37°C in a Hank's balanced salt solution (HBSS) containing 0.1% trypsin and 0.05% DNase. After 30 min of incubation, the tissues were mechanically dissociated in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and 0.58 g/L L-glutamine. This medium was supplemented with 100 μg/mL transferrin, 25 μg/mL insulin, 10 μg/mL putrescin, 5 ng/mL sodium selenite and 6.3 ng/mL progesterone (all from Sigma, St Quentin Fallavier, France). Viable cells were counted using trypan blue cell exclusion and plated at a density of 500 000 cells/well on 24-well polyornithine-coated culture plates. Cells were grown for 5 days, alone or in the presence of adenovirus, in a humidified incubator at 37°C in 5% CO_2/90% air atmosphere.

Adenoviral infections

To determine the adenoviral transduction efficacy of embryonic dopaminergic cells, three culture wells were infected with 25 pfu of Ad-βgal, and the number of tyrosine hydroxylase immunopositive (TH+) cells expressing β-galactosidase was assessed 48 h after infection (six fields were counted per well).

To investigate for neuroprotection against 6-OHDA, nine culture wells were infected with Ad-βgal (five at MOI 25, four at MOI 50), 11 with Ad-SOD (seven at MOI 25, four at MOI 50), five with Ad-GPx (all at MOI 25), and eight with empty vectors (four at MOI 25, four at MOI 50). Eight control wells were uninfected.

Twenty-four hours after plating, the cell medium was removed, and replaced with DMEM containing appropriate dilutions of each adenovirus. After 45 min of incubation, the adenoviral solutions were replaced by fresh culture medium. Non-infected cells were incubated in parallel in virus-free DMEM.

6-OHDA toxicity

Two days after adenoviral infection, 50 μM 6-OHDA (Sigma) or PBS (for controls) was added to cultures for 2 h. All wells but two

per group were treated with the 6-OHDA toxin. The viability of dopaminergic cells was assessed using TH immunohistochemistry and counting of the TH+ cells. All the TH+ cells were counted in each well. The percent viability was calculated as the ratio of TH+ cells in 6-OHDA-treated cultures to that in non-treated cultures.

Immunocytochemistry

TH immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA) in 0.1 M PBS, and incubated in 0.1 M PBS containing 10% goat serum and 0.1% Triton X-100 (blocking solution) for 1 h. Cells were then incubated overnight in the blocking solution containing a rabbit polyclonal anti-TH antibody (Jacques Boy 1 : 2000). The anti-TH antibody was detected using goat anti-rabbit Ig and avidin-biotin peroxidase complex (ABC kit, Vector) with 3,3′-diaminobenzidine (DAB) as the chromogenic substrate.

SOD immunocytochemistry

Cells were fixed with 4% PFA in 0.1 M PBS, and incubated in 0.1 M PBS containing 10% swine serum and 0.1% Triton X-100 (blocking solution) for 1 h. Cells were then incubated overnight in the blocking solution containing swine polyclonal anti-human SOD1 antibody (Valbiotech, Abcys, Paris, France, 1 : 500). The anti-SOD1 antibody was detected using swine anti-sheep/goat Ig (Amersham, Orsay, France) and avidin–biotin peroxidase complex (ABC kit, Vector) with 3,3′-DAB as the chromogenic substrate.

Double immunofluorescence TH/SOD1

Cultures were fixed with 4% PFA in PBS for 10 min. The cells were washed with 0.1 M PBS, and then incubated in a blocking solution (10% horse serum, 10% rabbit serum, and 0.1% Triton in 0.1 M PBS) for 1 h. They were then incubated overnight with sheep/goat polyclonal anti-human SOD1 antibody (Valbiotech, 1 : 500) and murine monoclonal anti-TH antibody (Boeringher, 1 : 300) diluted in the blocking solution. The anti-TH antibody was detected using horse anti-mouse Ig (ABC kit, Vector) and streptavidin–biotin phycoerythrin complex (1 : 150, Amersham). For detection of anti-human SOD1 antibody, cells were incubated with rabbit FITC-conjugated anti-sheep Ig (Biosys, Biovalley Marne La Vallee, France, 1 : 300).

Double staining X-gal/TH

Cells were fixed in PBS containing 4% PFA, and then incubated for 2 h at 37°C in a PBS solution containing 0.4 mg/mL of 5-bromo-4-chloro-3-indoyl-β-D-galactosidase (X-gal) substrate (Appligene, Illkirch, France) with 4 mM potassium ferricyanide (Sigma), 4 mM potassium ferrocyanide (Merck, VWR International, Strasbourg, France) and 4 mM MgCl2 (Merck).

The cultures were washed with 0.1 M PBS, and processed for TH immunocytochemistry as described above.

SOD enzymatic activity (NBT assay)

SOD activity was determined by gel electrophoresis followed by nitroblue tetrazolium (NBT) staining. Forty-eight hours after infection, Nonidet P-40 extracts were prepared from adenovirus-treated or non-treated cultures. Cell extracts were loaded on a 15% non-denaturing polyacrylamide gel, and electrophoresis was performed at 100 V. SOD activity was revealed by soaking the gel in distilled water containing 0.3 mM NBT and 0.26 mM riboflavin (20 min, room temperature) followed incubation in 90 mM tetramethylethylenediamine (TEMED) (for 20 min at room temperature).

Statistical analysis

The intergroup differences between the survival rates of TH+ cells were compared using a two-factor analysis of variance (ANOVA): virus × mutiplicity of infection (MOI).

Results

To analyse the neuroprotective potential of dopaminergic SOD1 gene transfer against (6-OHDA)-induced oxidative stress, we determined the survival rate of primary culture dopaminergic neurons infected with recombinant adenoviruses expressing antioxidative enzymes and exposed to neurotoxic concentrations of 6-OHDA.

Expression of functional enzymes in mesencephalic primary cultures

To investigate the adenoviral infection rate of embryonic dopaminergic neurons, we infected mesencephalic primary culture cells with the control adenovirus encoding *Escherichia coli* LacZ (Ad-βgal) at MOI of 25. Both dopaminergic and non-dopaminergic were efficiently transduced. Co-labelling experiments using combined X-gal cytochemistry and TH immunocytochemistry showed that 57.5 ± 5.75 of the TH+ neurons were transduced (Fig. 1).

To determine if Ad-SOD1 was able to direct expression of a functional human SOD1 protein in the cells, we infected the cultures with 25 MOI of vector and investigated the level of SOD1 enzymatic activity 2 days later in adenoviral-infected and non-infected cultures either exposed or not exposed to 6-OHDA. Protein extracts from the mesencephalic cells were run on gels, and SOD activity was revealed by NBT staining. This assay, which is based on the determination of the level of superoxide quenching in the gel, allows a semiquantitative analysis of SOD enzymatic activity. As endogenous rat SOD and human exogenous SOD could not be discriminated by their different mobilities in the gel, total SOD activity was quantified in the (Ad-SOD1)-infected cells and compared to that of the control (Ad-βgal)-infected and uninfected cells (Fig. 2a). Cell infection with 25 MOI of Ad-SOD1 nearly doubled the SOD enzymatic activity of cultures. SOD activities in non-infected cells and in cells infected with the control adenovirus were the same. The increased SOD activity in (Ad-SOD1)-infected cultures was thus due to the production of the recombinant human SOD1 protein in the transduced neurons. In addition, the 6-OHDA treatment had no significant effect on the level of SOD activity in any of the conditions tested (Fig. 2b).

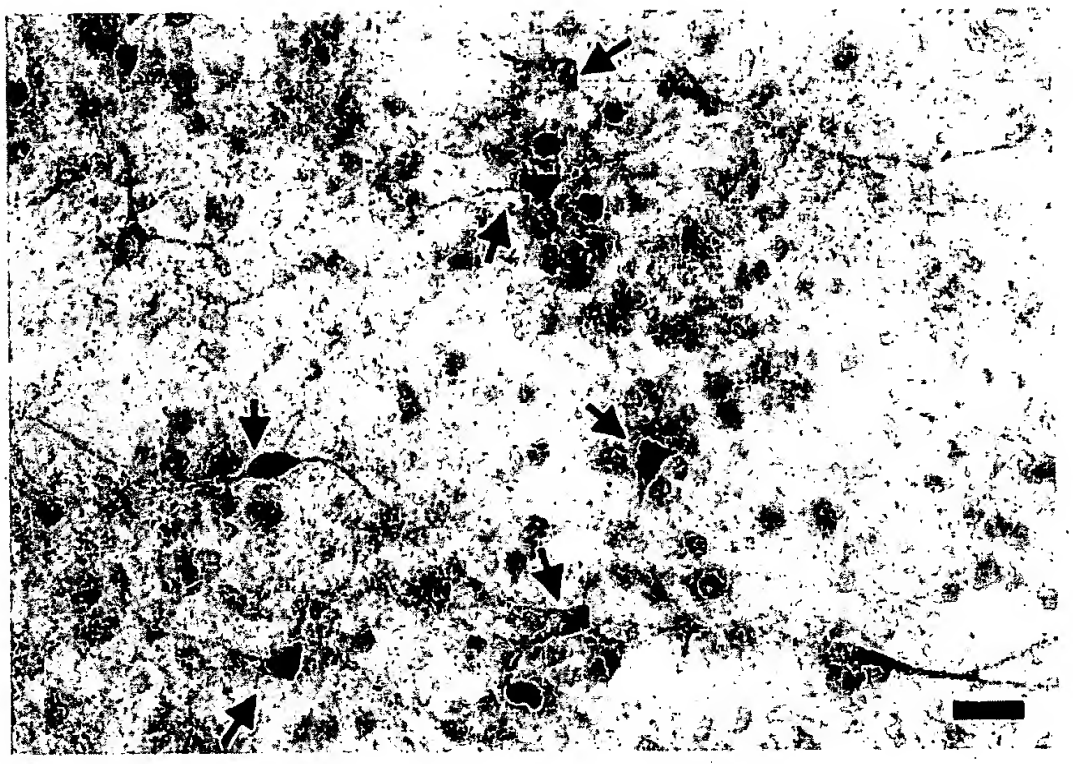

Fig. 1 Efficacy of adenoviral transduction in rat embryonic dopaminergic cultures. Combined X-gal cytochemistry and TH immunocytochemistry in mesencephalic cultures infected with 25 MOI of Ad-βgal. Expression of β-galactosidase was detected both in dopaminergic (TH+) and non-dopaminergic cells. 57.5 ± 5.7 TH+ neurons were efficiently transduced with Ad-βgal. Arrows, TH+ neurons expressing β-galactosidase (Xgal/TH double-labelled cells); arrowheads, non-transduced TH neurons. Scale bar = 30 μm.

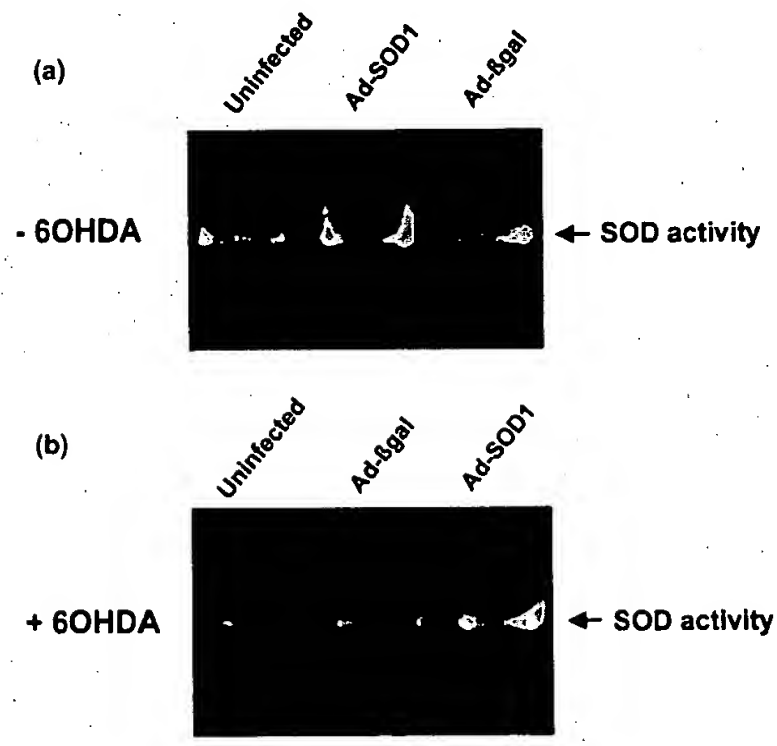

Fig. 2 SOD enzymatic activity. Gel electrophoresis of protein extracts from primary culture rat mecencephalic cells non-infected or infected with Ad-SOD1 or Ad-βgal (MOI 25). SOD enzymatic activity was determined using the NBT assay in (a) non-treated cells and in (b) 6-OHDA treated cells. The intensity of the band corresponding to SOD activity was nearly double that in cells infected with Ad-SOD1.

We then tested whether the recombinant human SOD1 protein was produced in the dopaminergic neurons (about 5% of the cells). Immunostaining specific for the human SOD1 protein was detected in the soma and neuronal processes of mesencephalic cells infected with a series of concentrations of Ad-SOD1 (Figs 3a–d). We detected no marked difference in the transduction efficacy between 25 and 75 MOI, but a slight viral toxicity was apparent at the latter dose. Co-labelling experiments using combined immunocytochemistry for human SOD1 and rat TH showed that the human protein was present in neurons either expressing or not expressing TH. Fig. 3(e–h) illustrate some TH+ neurons expressing the human SOD1 protein.

Neuroprotective effect of SOD1 overexpression

Exposure of mesencephalic cells to 50 μM 6-OHDA resulted in the death of about 65% of the TH-positive neurons. To investigate the protective effect of SOD1 overexpression on dopaminergic neurons, cultures were treated with Ad-SOD1 (MOI 25 and 50) two days before exposure to 6-OHDA. The viability of (Ad-SOD1)-infected cells was compared to that of non-infected cells, and of cells infected with Ad-βgal or empty control adenoviruses (Fig. 4). There was no significant difference between the viability of either (Ad-βgal)- or empty vector-infected cells, and therefore values were pooled into a single control virus group (mock). The two-factor analysis of variance (ANOVA) revealed a significant global effect of the adenoviral treatment ($p = 0.0012$) and a lack of effect of the viral MOI ($p = 0.63$). Since there was no significant 'virus–MOI' interaction ($p = 0.42$), comparisons were done between the virus-infected and non-infected groups, independently of the dose that was used (Fig. 4a). Cell transduction with the Ad-SOD1 vector resulted in a significant increase in cell viability compared to mock-infection (54% of increase, $p = 0{,}0003$). Cell viability of (Ad-SOD1)-treated cells was also significantly higher than that of uninfected cells (27.4% of increase, $p < 0.038$) (results are illustrated in Fig. 5).

The viability of Ad-GPx infected cells (MOI 25) was then compared to that of uninfected and mock-infected viruses.

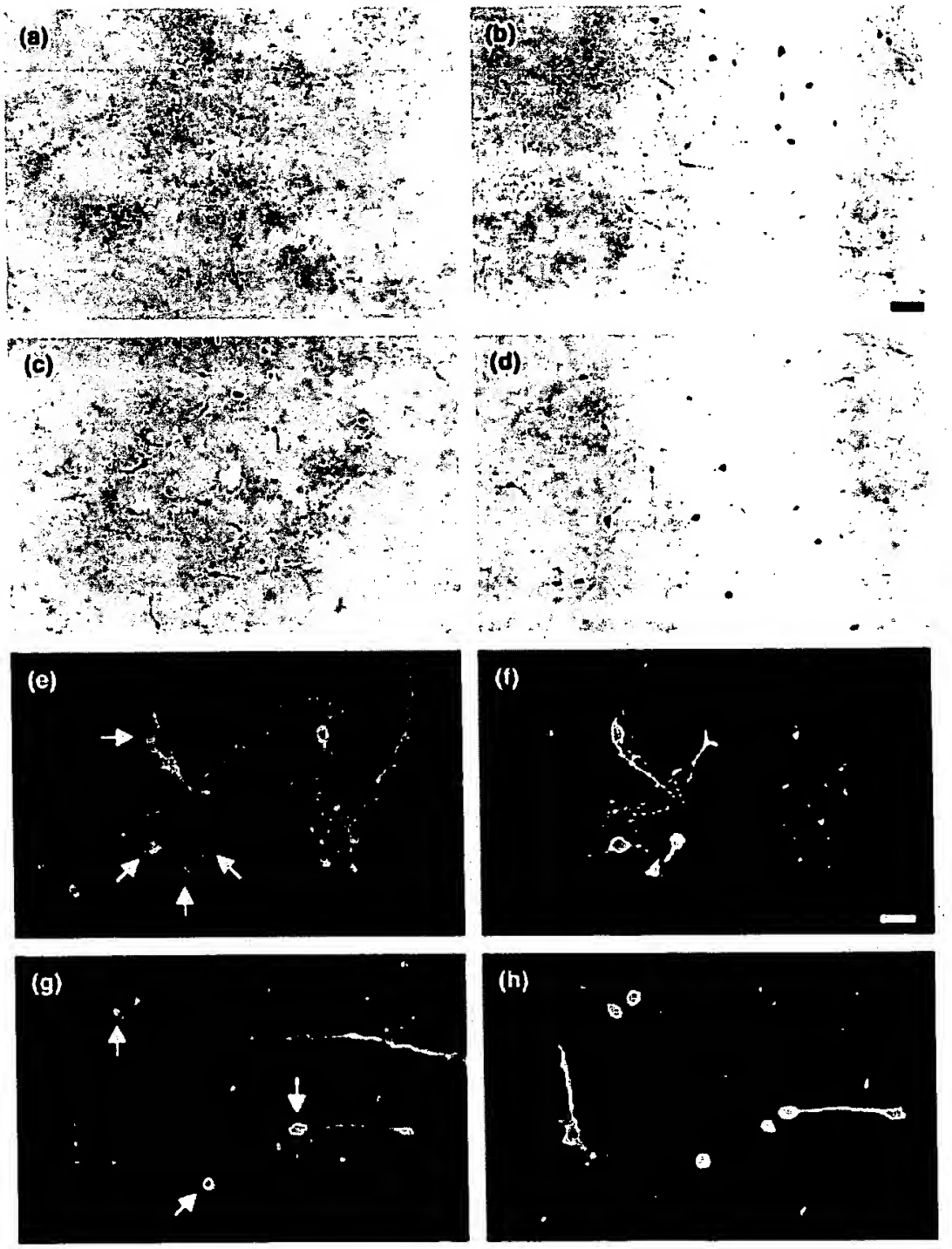

Fig. 3 Human SOD1 expression in (Ad-SOD1)-infected mesencephalic cells. (a–d) Immunocytochemical detection of the human SOD1 protein in primary culture rat mesencephalic cells (a) non-infected, (b) infected with 25 MOI of Ad-SOD1, (c) infected with 50 MOI of Ad-SOD1, and (d) infected with 75 MOI of Ad-SOD1. Scale bar = 100 μm (e–h): Co-labelling experiments showing TH+ dopaminergic neurons expressing the human SOD1 (hSOD1) protein. (e–g) phycoerythrin, red, TH. (f–h) fluorescein, green, human SOD1. White arrows: hSOD1/TH double-labelled dopaminergic cells. Scale bar = 30 μm.

No significant increase in resistance to 6-OHDA was found when cells overexpressed GPx (one factor ANOVA, $p > 0.2$) (Fig. 4b). In contrast, cultures infected with 25 MOI of Ad-SOD1 were significantly more resistant to 6-OHDA than those infected with the control viruses (mock-infected). We did not further test the effect of infection with Ad-GPx at 50 MOI since no marked increase in the transduction efficacy of adenoviral vectors was found at this concentration (see also Barkats *et al.* 2000). The functionality of this adenoviral vector was previously evidenced using RT-PCR and enzymatic activity assays in 293 cells (Barkats *et al.* 2000).

Discussion

6-OHDA, which is commonly used to induce PD in experimental animals, is thought to cause dopaminergic cell death via a free radical mechanism. Using primary cultures of rat ventral mesencephalon, we examined the effect of SOD1 overexpression on the survival of dopaminergic neurons exposed to 6-OHDA. To overexpress the antioxidative enzyme in neurons, we used a gene delivery method based on recombinant adenoviruses. These viral vectors are among the most efficient for transducing postmitotic cells like neurons both *in vitro* and *in vivo* (Akli *et al.* 1993; Bajocchi *et al.* 1993; Davidson *et al.* 1993; Le Gal La Salle *et al.* 1993).

Co-labelling experiments combining Xgal and TH stainings in (Ad-βgal)-infected cultures revealed the transgene expression within both dopaminergic and non-dopaminergic cells. The percentage of dopaminergic neurons that were transduced after exposure to 25 MOI of Ad-βgal went beyond 50%. Production of the human SOD1 protein was evidenced in the mesencephalic cultures using a semiquantitative

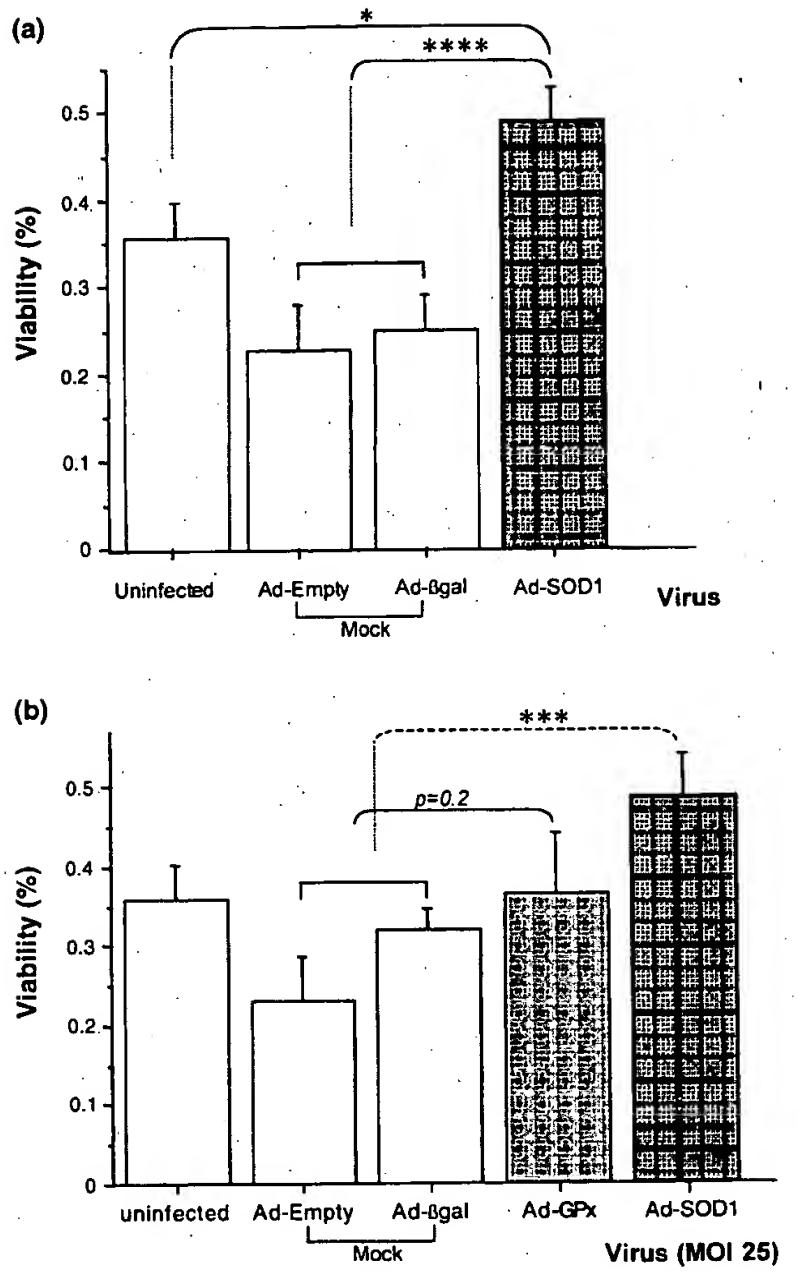

Fig. 4 Ad-SOD1 infection increased resistance to 6-OHDA cytotoxicity. Cytotoxic response of primary culture dopaminergic cells to 6-OHDA. About 65% of TH+ cells were killed by a 24-h exposure to 50 μM 6-OHDA. (a) Comparison of the dopaminergic cell viability between Ad-SOD1-infected cells (Ad-SOD1) and cells either uninfected or infected with control adenoviruses (mock) using a two-factor analysis of variance (ANOVA): 'viral dose' (25, 50) × 'viral treatment' (Ad-SOD1, Ad-βgal, mock, uninfected). The viability of TH+ cells was significantly increased in Ad-SOD1-infected cultures. (*$p < 0.05$, ****$p < 0.0001$, two-factor analysis of variance, *post-hoc* Fisher's test). (b) Analysis of cell viability in mesencephalic cultures infected with 25 MOI of Ad-GPx. Results shows no apparent neuroprotective effect of Ad-GPx infection, while Ad-SOD1 infected cells were significantly more resistant to 6-OHDA than mock-infected cells (***$p < 0.001$, one-factor analysis of variance, *post-hoc* Fisher's test). Cell viability was assayed by counting all TH+ cells per culture well, and results are expressed as percent viability, corresponding to the number of 6-OHDA-treated cells relative to untreated cells.

enzymatic assay and immunocytochemistry specific for the recombinant protein. Although we did not determine the exact number of the (Ad-SOD1)-transduced TH cells, it might be comparable to that of colabelled Xgal/TH cells (approximately 50%) since similar promoters (RSV) were used in both Ad-βgal and Ad-SOD1 constructions. The semiquantitative NBT assay showed that the intracellular SOD enzymatic activity in extracts of mesencephalic cells infected with 25 MOI Ad-SOD1 was nearly double that in uninfected cell extracts. This result suggests that cell infection with Ad-SOD1 led to a more than two-fold increase in cytoplasmic antioxidative activity (since only approximately 50% of dopaminergic neurons could be transduced with 25 MOI of adenoviral vectors).

Infection of mesencephalic cells with Ad-SOD1 prevented the death of a significant number of dopaminergic neurons exposed to 6-OHDA. The percent survival of TH+ neurons in cultures infected with Ad-SOD1 was significantly increased compared to controls treated with Adβgal or empty vectors (approximately two-fold). The difference with non-infected cells was less pronounced probably due to a residual toxicity of the adenoviral vector. The SOD1 enzyme is not secreted, and is exclusively synthesized in the cytoplasmic cell compartment. This suggests that only the (Ad-SOD1)-infected dopaminergic cells could be protected from (6-OHDA)-induced neurotoxicity. As only half the TH+ cells were transduced by adenoviral vectors, our results suggest that a large majority of the (Ad-SOD1)-transduced dopaminergic neurons were protected from 6-OHDA.

Potential trophic effects mediated by glia were insignificant in our experiments since as less than 1% of the cells in culture (under serum-free conditions) were glial cells (Frodl *et al.* 1994). A non-specific protective effect of treatment with adenoviral vectors, conceivably induced by a stress adaptative response of the cells, can also be excluded since no protective reaction against 6-OHDA toxicity was detected with control adenoviruses (empty vectors or vectors expressing β-galactosidase). Therefore, our results strongly suggest that the intraneuronal overexpression of SOD1 can inhibit dopaminergic cell death in (Ad-SOD1)-infected cultures.

The mechanisms underlying prevention of dopaminergic cell death by SOD1 overexpression are related to the sensitivity of dopaminergic neurons to (6-OHDA)-mediated oxidative stress. Earlier *in vivo* studies show that 6-OHDA injected into the striatum of rodents is transported into dopaminergic neurons where it is oxidized into reactive oxygen species (Kumar *et al.* 1995). Superoxide generated by 6-OHDA (Heikkila and Cohen 1973) can further cause dopaminergic cell death by interacting with nitric oxide to form the highly reactive peroxynitrite radical, or it can react with iron or copper to generate hydroxyl radicals. SOD, which detoxifies free radicals, may have a protective effect on dopaminergic neurons by scavenging (6-OHDA)-generated superoxide. Indeed, when overexpressed in transgenic animals, the superoxide-scavenging SOD1 protein was

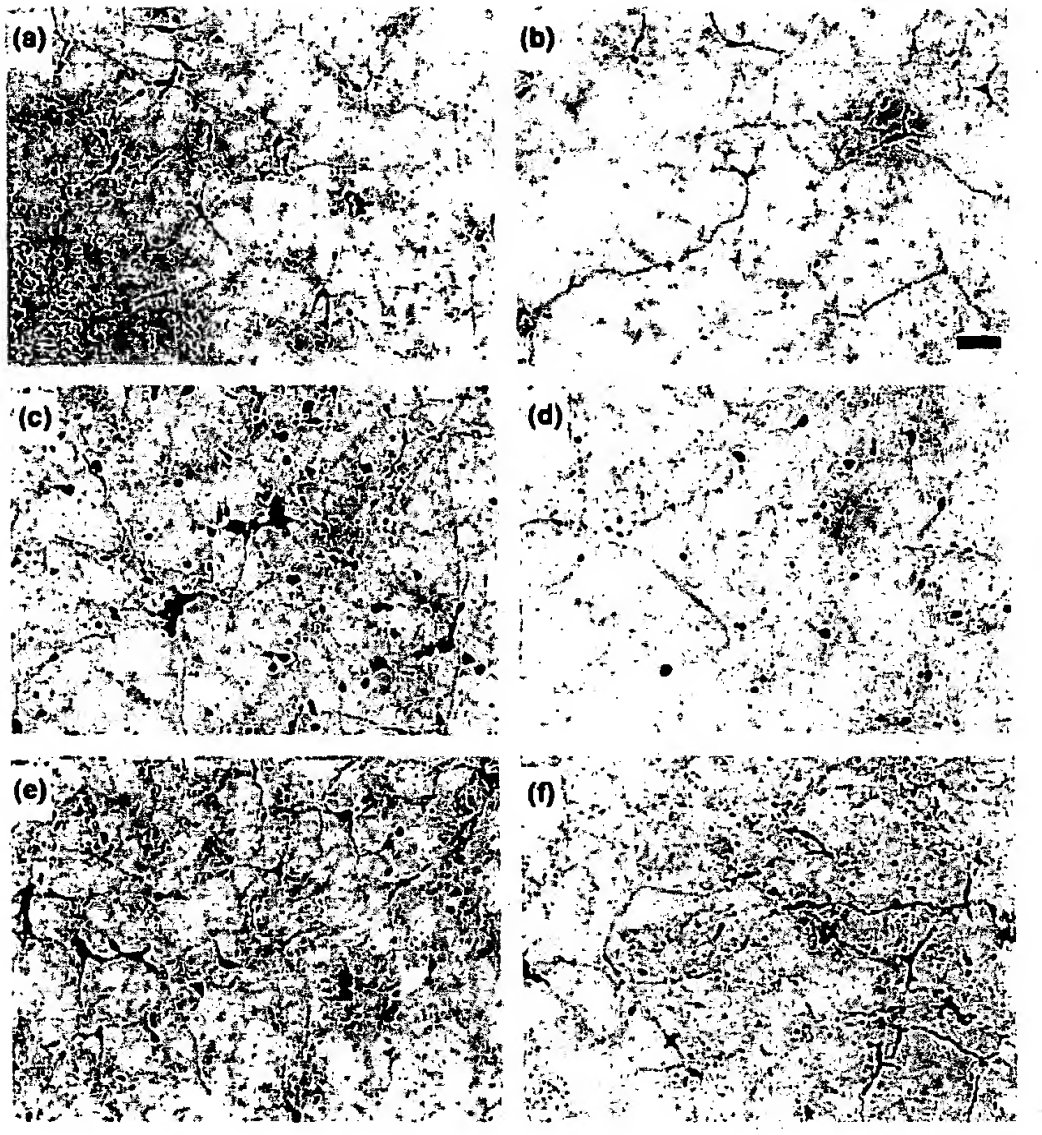

Fig. 5 Resistance of Ad-SOD1-transduced dopaminergic cells to 6-OHDA. Dopaminergic cells were evidenced using TH-immunostaining. Cells received (a, c and e) no. 6-OHDA treatment (b, d and f) 50 μM 6-OHDA (24 h exposure). (a and b) Control uninfected mesencephalic TH+ cells. (c and d) Mesencephalic cells infected with 25 MOI of Ad-βgal (double-staining Xgal/TH). (e and f) Mesencephalic cells infected with 25 MOI of Ad-SOD1. More than half of the dopaminergic cells have degenerated in uninfected (b) and Ad-βgal infected (d) cells. Note the neuroprotective effect of SOD1 overexpression in Ad-SOD1 infected cultures (f). Scale bar = 100 μm.

reported to the prevent the death of dopaminergic neurons induced by intracerebroventricular injection of 6-OHDA (Asanuma *et al.* 1998).

It has also been suggested that the neurotoxin 6-OHDA exerts its toxic effects on dopaminergic cells by production of H_2O_2 (Perumal *et al.* 1989). However, we did not find any significant effect of the overexpression of the H_2O_2-scavenging enzyme GPx in cultures infected with Ad-GPx before exposure to 6-OHDA. The lack of neuroprotective effect of GPx was unexpected because a partial protection of dopaminergic neurons against (6-OHDA)-induced neurotoxicity has been described in transgenic mice overexpressing GPx (Bensadoun *et al.* 1998). The discrepancy may result from a decreased availability of reduced glutathione (GSH) in our primary culture model. Indeed, the level of oxidized glutathione (GSSG) increases substantially after treatment with 6-OHDA (Spina *et al.* 1992). The concomitant decrease of GSH (Perumal *et al.* 1989) and/or glutathione reductase (GR) may therefore have limited the neuroprotective action of GPx in our experiments. This idea was also discussed by Bensadoun *et al.* (1998). The question of whether detoxification of H_2O_2 may contribute to reducing (6-OHDA)-mediated neurotoxicity could therefore be investigated by cotransducing cells with viral vectors encoding both GPx and GR, or GPx and the brain-derived neurotrophic factor (BDNF) which is known to induce the increase of GR activity (Spina *et al.* 1992).

In a previous study using *ex vivo* gene transfer technology, we showed that overexpression of SOD1 in dopaminergic neurons prior intrastriatal grafts in hemiparkinsonian rats increased the functional recovery of the animals (Barkats *et al.* 1997). This study suggested that free radicals were important agents in the dopaminergic cell death induced by the grafting procedure. Here, we further demonstrate the great neuroprotective potential of recombinant SOD1 adenoviral vectors for dopaminergic neurons in culture exposed to the 6-OHDA neurotoxin. Since it is clear that free radical homeostasis is extremely important for the survival of dopaminergic neurons, antioxidant gene transfer based on viral vector methodologies similar to those used in our study allows direct *in vivo* application of neuroprotective strategies in experimental models of neurodegenerative diseases. Scavenging enzymes, like SOD1, delivered directly to degenerating dopaminergic neurons by means of viral vectors, have enormous potential as neuroprotective agents in these experimental diseases. These antioxidant-based gene transfer strategies may reveal themselves more efficient than those using growth factors such as the glial cell line-derived

neurotrophic factor (GDNF) or the BDNF. Indeed, a carboxyfullerene derivative (the C3 potent antioxidant) has recently been reported to outperform the highly protective effect of GDNF in the 6-OHDA model (Lotharius *et al.* 1999). Moreover, both GDNF and BDNF were recently suggested to act on dopaminergic neuron survival by activating the antioxidative enzyme systems (Spina *et al.* 1992; Chao and Lee 1999).

In conclusion, our study demonstrates the potential of an adenoviral vector encoding the human SOD1 to prevent (6-OHDA)-induced dopaminergic cell death by increasing the antioxidant potential of neurons. As 6-OHDA could act as an endogenous neurotoxin in PD, and because there is evidence for the contribution of oxidative stress in this disease, these results may be of significance for gene therapy of PD.

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